

Passive Permeability of the Human Placenta 'In-vivo'

Murray D Bain

Presented for the Degree of Doctor of Medicine  
University of Edinburgh

1991



Acknowledgements.

I should like to thank all the staff of the now closed section of Perinatal and Child Health at the Clinical Research Centre, Harrow for their interest in and encouragement with this study; I should like to thank especially Dr MJ Landon for his unflinching availability at times of need, and Miss D Copas for her technical help. My thanks also go to Prof TE Stacey, whose original idea provided the basis for this study.

This study would not have been possible without the co-operation of those patients who agreed to participate in the study and who must remain the unsung heroes of this study.

I certify that the work contained in this thesis was performed by myself except where stated otherwise,

Murray D Bain.

Abstract.

Transfer across the placenta between mother and foetus underlies normal foetal growth and development. Although passive diffusion is the simplest of the several mechanisms of transfer and despite its potential importance for foetal growth, there have been few 'in-vivo' measurements of passive permeability in the human. To provide such data passive permeability was measured 'in-vivo' in 13 pregnancies scheduled for delivery by elective caesarean section.

Four different sized, inert, and hydrophilic, molecular permeants were used; mannitol, lactulose, CrEDTA, and inulin. A bolus and constant infusion were used to create a maternal-foetal concentration gradient. The subsequent neonatal urinary excretion of the permeants was measured and equalled the net transfer to the foetus as the permeants were cleared wholly and solely by renal excretion. The passive permeability of the placenta was calculated by dividing the net flux by the mean transplacental concentration gradient.

Permeability measurements for mannitol and inulin were undertaken in 6 pregnancies, and for all four permeants in 7 pregnancies. Placental clearance (passive permeability of the whole placenta) was (mean  $\pm$  S.E. of mean)  $8.1 \pm 0.7$  ml.min<sup>-1</sup> for mannitol,  $6.3 \pm 0.8$  for lactulose,  $3.7 \pm 0.5$  for CrEDTA, and  $0.92 \pm 0.07$  for mannitol. The corresponding passive permeabilities per unit weight placenta were  $13.52 \pm 1.32 \times 10^{-3}$  ml.min<sup>-1</sup>.g<sup>-1</sup> for mannitol,  $10.84 \pm 1.55 \times 10^{-3}$

for lactulose,  $6.33 \pm 0.84 \times 10^{-3}$  for CrEDTA and  $1.53 \pm 0.11 \times 10^{-3}$  for inulin.

The measured placental clearances were found to be low compared with the blood flows on either side of the placenta ( $300 - 500 \text{ ml.min}^{-1}$ ), indicating the placenta to be a significant barrier to passive diffusion between maternal and foetal vascular compartments. While such a barrier will facilitate foetal homeostasis independent of that of the mother, the cost will be limited transfer by passive diffusion between mother and foetus. Where the foetal requirement exceeds this low rate of transfer, special transfer mechanisms will be necessary to ensure adequate foetal supply. This was concluded to be the case for glucose, the principal foetal metabolic fuel, whereas passive diffusion alone appeared adequate for the foetal excretion of creatinine. The 'in-vivo' permeability data allowed direct validation of the technique of 'in-vitro' perfusion of the isolated human placental lobule which is widely used to obtain measurements of placental transfer that had previously been unobtainable. This showed that 'in-vitro' perfusion distorted the transfer characteristics of the placental membrane despite previous studies demonstrating metabolic and structural integrity of the preparation. The 'in-vivo' data also demonstrated that the documented variation in placental permeability between the species extends to man, making it difficult to find a single animal model that encompasses all aspects of human placental transfer.



## Table of Contents.

	Page No.
<u>Chapter 1.</u>	<u>13 - 35</u>
A General Introduction to Placental Transfer	
- Historical background	
- Significance of placental transfer	
- Placental structure and development	
- Mechanisms of placental transfer	
- Animal studies of placental permeability	
<u>Chapter 2.</u>	<u>36 - 43</u>
Passive permeability studies of the human placenta	
- 'In-vitro' studies	
- 'In-vivo' studies	
- Summary of background	
- Aims of the study	
<u>Chapter 3.</u>	<u>44 - 86</u>
Methodology	
- Clinical	
- Analytical	
<u>Chapter 4.</u>	<u>87 - 99</u>
Mathematical theory and analysis	
- Derivation of permeability constants	
- Measurement of molecular size	
- Compartmental analysis	
- Statistical analyses	

Chapter 5.100 - 128

## Results

- Patient details
- Blank values
- Molecular size of CrEDTA
- Serum profiles
- Determined values for placental clearance
- Correlates of placental clearance
- 'In-vitro' versus 'in-vivo' placental permeability
- Volumes of permeant distribution

Chapter 6.129 - 166

## Discussion

- Sources of error
- Comparison of with other available data
  - (1) Molecular size of CrEDTA
  - (2) Placental clearances
- Physiological implications of the data
  - (1) Nature of the placental barrier
  - (2) Functional consequences of the placental barrier

Chapter 7.167 - 169

## Conclusions

References.170 - 180Bibliography for chapter 1.180

## List of Plates and Tables

- Plate 1. Electron micrograph of the human placental membrane
- Plate 2. Urine collecting apparatus
- Table 1. Some species differences in placentation
- Table 2. 'In-vitro' placental permeability data for some animal species
- Table 3. The two different infusion regimes
- Table 4. Reasons for delivery by elective caesarean section
- Table 5. Maternal clinical details in the 13 patient-studies
- Table 6. Some properties of the test molecules
- Table 7. Neonatal details for group 1 patient-studies
- Table 8. Permeability data in group 1 patient-studies
- Table 9. Neonatal details for group 2 patient-studies
- Table 10. Permeability data in group 2 patient-studies
- Table 11. Ratio of 'in-vivo' and 'in-vitro' permeabilities, and the ratio of coefficients of free diffusion
- Table 12. Ratio of clearances and permeant concentrations in amniotic fluid and other body fluids
- Table 13. Placental clearances excluding and including amniotic fluid content
- Table 14. Comparison with 'in-vitro' perfusion data
- Table 15. Placental characteristics determining foetal-maternal exchange of various substrates

## List of Figures.

- Fig 1. Embryology and anatomy of the human placenta
- Fig 2. The placental barrier and its transport mechanisms
- Fig 3. Permeability and structure of the cell membrane
- Fig 4. Placental permeants used in the current study
- Fig 5. Standard curve of Heyrovsky reaction with inulin and lactulose standards
- Fig 6. Column chromatography of infused and excreted inulin
- Fig 7. Neonatal urinary excretion of inulin activity in 2 control infants and 6 patient-studies
- Fig 8. Column chromatography of neonatal urine from a control study
- Fig 9. Column chromatography of the representative pooled sample of a total neonatal urine collection (group 1 patient-study)
- Fig 10. Column chromatography of representative pooled samples of total neonatal urine collections (group 2 patient-studies)
- Fig 11. Column recovery of eluted inulin
- Fig 12. Urinary excretion of mannitol and inulin in 3 of the group 1 patient-studies
- Fig 13. Column chromatography of sequential neonatal urine samples
- Fig 14. Background cross-reacting material in maternal and neonatal urine
- Fig 15. Stability of the infused inulin

- Fig 16. Maternal and cord serum profiles from a group 1 and group 2 patient-study
- Fig 17. Chromatography of the terminal urine sample of the neonatal urine collection in 3 patient-studies
- Fig 18. Column chromatography for measurement of the  $K_{av}$  of CrEDTA
- Fig 19. Relationship between  $K_{av}$  and molecular radius allowing the determination of the effective molecular radius of CrEDTA
- Fig 20. Serum profiles for mannitol in group 1 patient-studies
- Fig 21. Serum profiles for inulin in group 1 patient-studies
- Fig 22. Serum profiles for mannitol in group 2 patient-studies
- Fig 23. Serum profiles for lactulose in group 2 patient-studies
- Fig 24. Serum profiles for CrEDTA in group 2 patient-studies
- Fig 25. Serum profiles for inulin in group 2 patient-studies
- Fig 26. Relationship between placental permeability and smoking habits
- Fig 27. Relationship between placental permeability and permeant molecular radius
- Fig 28. 'In-vivo' versus 'in-vitro' placental permeabilities for the sequentially perfused placentas

Fig 29. Placental permeabilities and permeability ratios for the 4 patient-studies where 2 permeants were studied sequentially 'in-vivo' and 'in-vitro' in the same placenta

Fig 30. Comparison of the 'in-vivo' human placental permeability data with equivalent data for other animal species

Fig 31. 'In-vivo' placental permeability data fitted to a uniporous model of the placental membrane

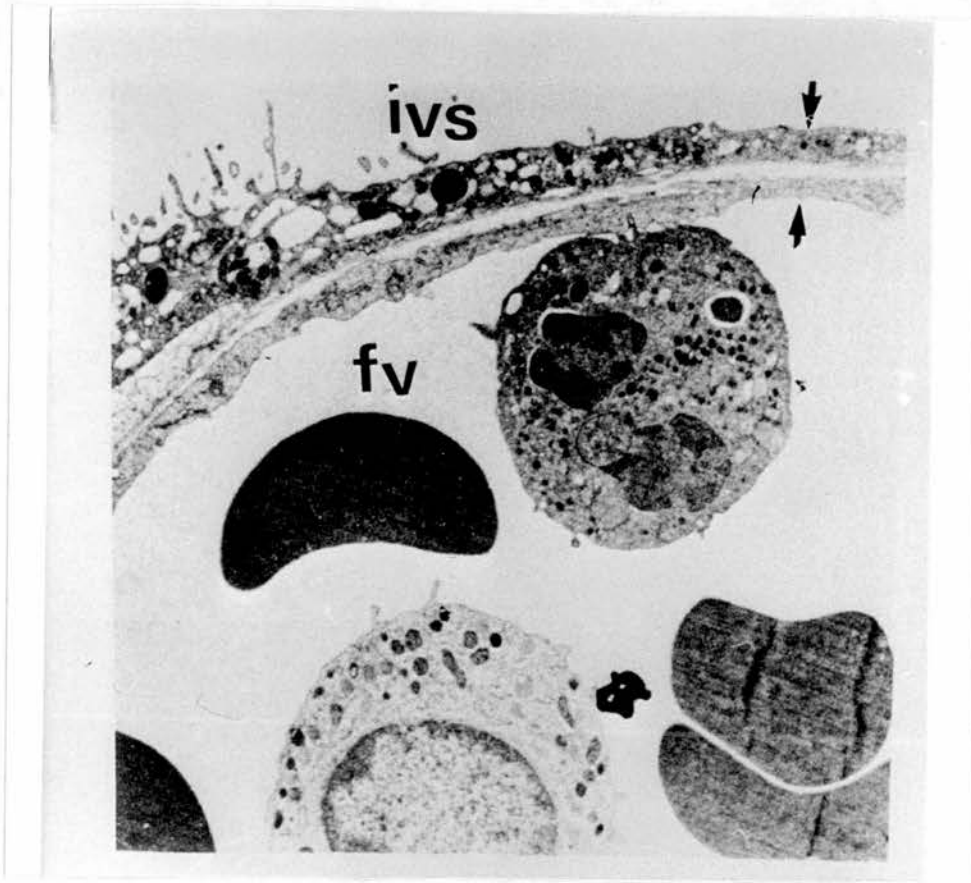


Plate 1. An electron micrograph of the human placental membrane with the vasculo-syncytial region indicated between the arrows (Courtesy of Prof TE Stacey). Ivs., intervillous space; fv., foetal blood vessel



Plate 2. Urine collecting apparatus



## Chapter 1.

## Placental Transfer - A General Introduction.

Historical Background.

The recognition of the importance of the placenta to the foetus may be traced to the beginnings of recorded history. The ancient Egyptians, for example, are believed by some authors to have held the placenta to be the seat of the 'External Soul', as a standard interpreted as representing the royal placenta of the Pharaohs was often carried in ceremonial processions before the reigning Pharaoh and was given special reverence. Although by ancient Greek times the three membranes, the chorion, the amnion, and the allantois were recognised, the true role of the placenta as the conduit of transfer from mother to foetus was first acknowledged by Aristotle (384 - 322 BC). He described the placenta as the source of foetal nutrition, the sanguineous pabulum, from the mother and is also credited with being the first to use the term chorion.

Our expanding understanding of placental function and physiology over the intervening centuries has depended on advances in other branches of science and has been crucially influenced by the prevailing views of the time. Thus Galen (circa 130 - 200 AD), the other major classical contributor to embryology, considered the maternal and foetal vessels to

be in continuity, and this view prevailed until the Renaissance (circa 1453 - 1628).

Arantius (1530 - 1589) recognised that the foetal and maternal circulations were not infact continuous and this was a necessary prelude to Harvey's (1578 - 1657) conclusions on the circulation and flow of blood from arteries to veins. It was only with Malpighi's (1628 - 94) published discription of capillaries in 1660 that the anatomical circuit between arteries and veins was complete and a means of separating foetal and maternal circulations identified. Despite Malpighi's observations, the Galenic view of an open continuity between the foetal and maternal vessels persisted in certain quarters well into the following century. A corollary of separate circulations was the necessity for transfer across the placenta and in 1674 Mayow (1640 - 1679) was able to suggest that the placenta served as the foetal lung. Over the next couple of centuries much work was done on comparative placental anatomy and there was rapid progress in midwifery, but little advancement in the study of placental function. The placenta during this period was seen as nurturing the growing foetus just as the roots nurture the growing plant, the only understandable analogy available at that time.

With the establishment of separate maternal and foetal circulations, further study of transfer between these two very different vascular compartments depended on advances in the physical and biological sciences. Necessary milestones in this direction included, the isolation of oxygen in 1774

(Priestly, 1733 - 1804), the concept of oxidative combustion (and hence the need for oxygen) as the source of energy for living tissues (Lavoisier, 1743 - 1794), the demonstration of carbon dioxide and oxygen in both arterial and venous blood in 1837 (Magnus, 1802 - 1870), the concept of tissue respiration with blood merely having a carrier role and not itself engaging in respiration or directly controlling tissue respiration (Traube, 1826 - 1894, Pfluger, 1829-1910), and Liebig's (1803 - 1873) apparatus allowing division of foodstuffs into carbohydrate, fat, and protein which led on to the identification of sugars and amino acids in the latter half of the nineteenth century. The invention of the spectroscope in the 1850's and its application to the blood pigments in the 1860's by Stokes allowed Zweifel in 1876 to demonstrate the placental transfer of oxygen. In that same year Turner could add strychnine, potassium cyanide, and alizarian dye as substances that cross the intact placenta 'in-vivo', and in 1888 Cohnstein and Zuntz showed the placental transfer of glucose in the guinea pig.

Our current understanding of the patterns and individual mechanisms of transfer across the placental membrane separating the maternal and foetal vascular compartments dates from 1941. In that year Dean first proposed the existence of an energy requiring membrane sodium pump in muscle and in 1952 Davson and Danelli proposed their now classic lipid bilayer as the structure for cell membranes which has provided the basis for modern membrane physiology. Also in 1952, Widdas suggested carrier mediated transfer in

the placenta to explain the greater permeability of the sheep placenta to glucose than to fructose.

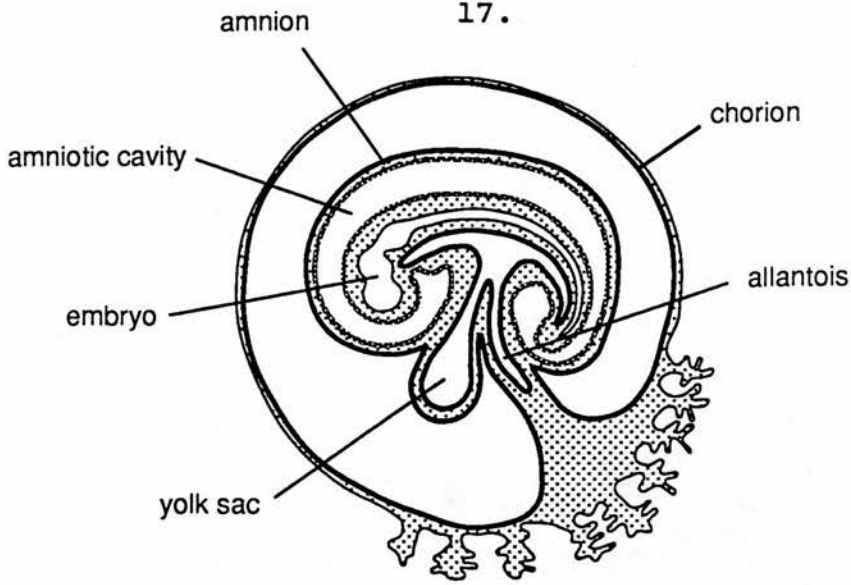
#### Significance of placental transfer.

The foetus is completely dependent on the mother for nutrition, respiration, and excretion. Underlying this exchange between mother and foetus is a pattern of transfer that provides the requirements for foetal growth at a time when the growth rate is proportionately faster than at any other time of life. The placenta provides this transfer. Where placental transfer is inadequate, foetal growth is compromised. In animals, foetal growth retardation can be induced by experimental reduction of maternal-foetal substrate exchange (Moll, 1985), and in man recent data from studies of uterine blood flow (Pearse & Steel, 1988), maternal vascular studies (Labarre, 1988) and the placental membrane itself (Teasdale & Jean-Jacques, 1988) all suggest inadequate placental transfer may be a major cause of intrauterine growth retardation, a significant cause of perinatal death and postnatal morbidity. Understanding how the pattern of placental transfer provides the necessary maternal-foetal exchange is thus fundamental to understanding placental function in health and disease.

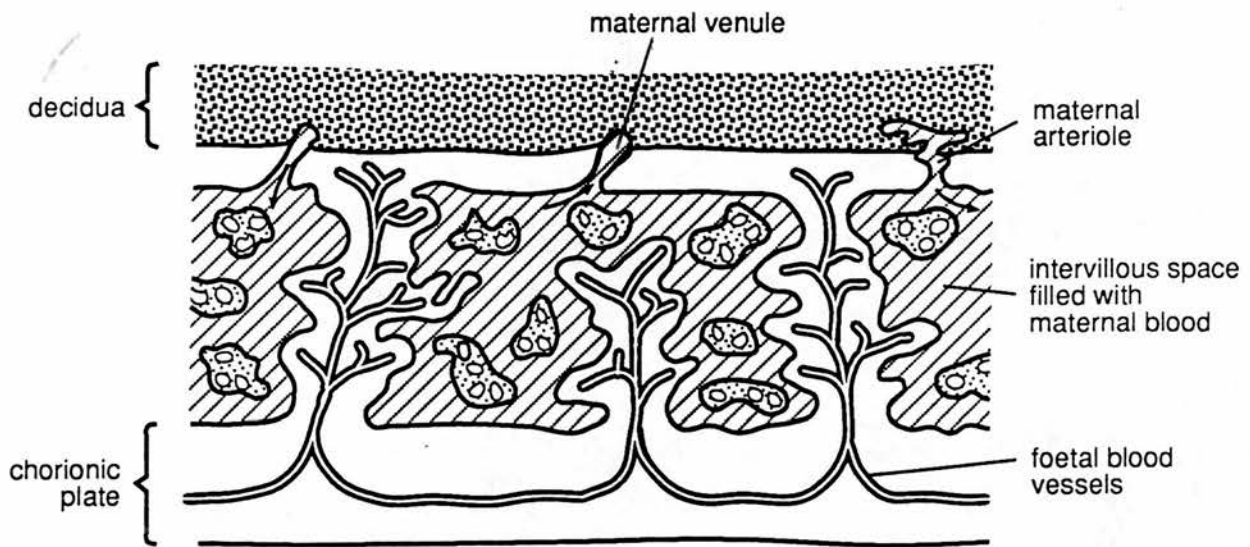
#### Development and structure of the human placenta.

##### Development.

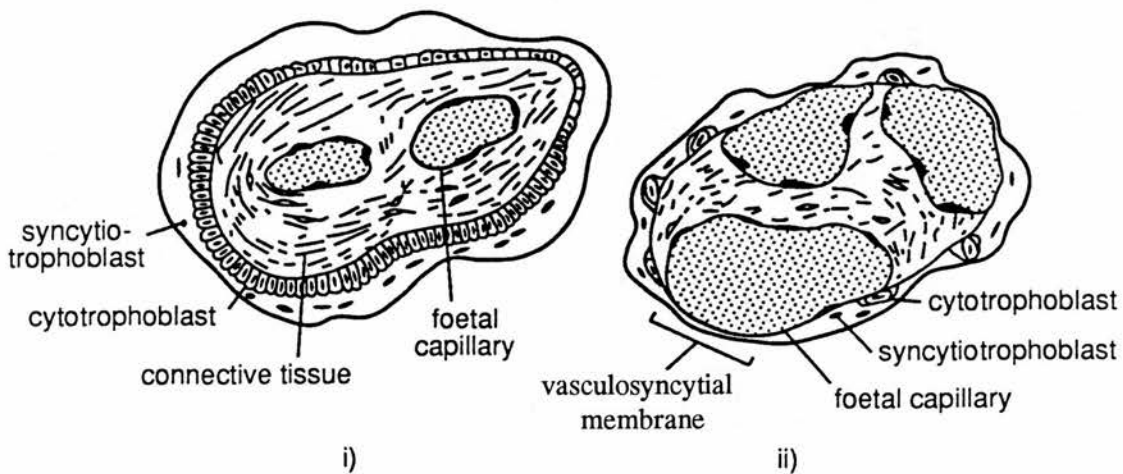
The development of the human placenta differs in significant ways from that of other animals so setting a



a) Embryology of the placenta



b) Vascular arrangement within the placenta



c) Histology of the placental villi in i) early and ii) late pregnancy

precedent for the differences in structure and function that make extrapolation from animal data to man so uncertain.

Faber and Thornburg (1983) list three phylogenetically different placentas in mammals which are developed from the chorion: chorionic (the earliest), choriovitelline (derived from the yolk sac) and chorioallantoic (derived from the allantois, an outgrowth from the gut caudal to the yolk sac). The chorionic placenta appears in all animals as the conceptus is embedding in the endometrium but is superseded by either of the other two types of placenta which are taken to represent phylogenetically more advanced types of placentation. Strictly, the chorion refers to the membrane consisting of an outer layer of trophoblast and an inner layer of non-vascular mesoderm.

The human placenta is usually classified as a chorioallantoic placenta though the chorion is infact the major foetal contributor to placental formation in man and this is more reminiscent of the chorionic type of placenta. The placenta in man forms from trophoblastic development, initially present over the whole of the chorionic sac, but which soon becomes confined to an area beneath the growing embryo, with regression elsewhere (Fig 1a). Though a yolk sac develops it does not form a choriovitelline placenta as in the rat or even persist as a functioning yolk sac placenta throughout gestation as in the guinea pig; and though an allantois also develops and even grows into the body stalk, it does not make contact with the chorion to form a true chorioallantoic placenta as in the sheep but its



vessels provide the necessary connection between the embryonic vascular channels and the developing placenta (hence its classification as a chorioallantoic placenta). The trophoblastic development forms bulges into the maternal endometrium, the so-called primary villi. As the trophoblastic development occurs the outer trophoblastic layer loses its intercellular boundaries to become a syncytium, the syncytiotrophoblast, while the deeper lying cells retain distinct cell boundaries, the cytotrophoblast. These primary villi acquire a connective tissue core from mesenchymal ingrowth and as they grow they destroy the adjacent maternal endometrium including blood vessels. The invading villi therefore come to lie surrounded by maternal blood with the syncytiotrophoblast in direct contact with the blood, an arrangement that is one of the characteristics of the functioning human placenta for the rest of gestation (fig 1b).

The syncytiotrophoblast, while being the only epithelial syncytium in man, is not the only syncytium, striated skeletal muscle for example being a multinucleate syncytium. The syncytial nature of skeletal muscle is presumably a specific adaption because both smooth and cardiac muscle are mono-nucleate and it is tempting to speculate that the syncytiotrophoblast has a similar and specific adaptive function. By its very nature, the syncytiotrophoblast has no paracellular pathways of transfer which are very important in other epithelia, for example the intestine.

During pregnancy the placenta continues to grow and

develop and between 28 weeks and term (40 weeks) the placenta doubles in weight from 300 g to 600 g while the foetus increases in weight by a factor of three from 1 kg to 3.5 kg. New villi are still being formed at term (Fox, 1979) though the branching process has substantially reduced the diameter of the villi to around  $40\ \mu\text{m}$ . The villous surface area also increases substantially during pregnancy. Aherne and Dunhill (Aherne & Dunnill, 1969) estimated the surface area of the syncytiotrophoblast exposed to the maternal circulation to be  $3.4\ \text{m}^2$  at 28 weeks, increasing to  $11\ \text{m}^2$  at term (excluding the microvilli). The syncytiotrophoblast is at its most attenuated in the terminal villi and it is here that the foetal and maternal blood are in closest apposition and it is here that most exchange is believed to take place.

#### Structure of the human placental membrane.

In early pregnancy the placental membrane between maternal and foetal vascular compartments consists of the three tissue layers: foetal capillary endothelium, mesoderm, and trophoblast. The cytotrophoblast layer of the trophoblast regresses after the second month and the syncytiotrophoblast becomes attenuated, from  $10\ \mu\text{m}$  to about  $1.7\ \mu\text{m}$  in late gestation (Boyd & Hamilton, 1967), so that most villi come to lie clothed in a reduced syncytiotrophoblast layer with only the occasional cytotrophoblastic cell persisting (Fig 1c). Other changes with gestation also serve to bring the two circulations into closer apposition: the foetal villous vessels increase in



diameter, they also move from a central to a more peripheral position, the microvilli covering the syncytial surface increase in number from 600 million.cm<sup>-2</sup> at the fourteenth week to 1200 million.cm<sup>-2</sup> at term (Ludwig, 1974), and in the late pregnancy there develop areas of focal attenuation of the syncytiotrophoblast, called vasculo-syncytial membranes, where there is an absence of nuclei and the syncytium appears particularly closely applied to the capillary basement membrane (Fig 1c). The exact functions of the microvilli and vasculo-syncytial membranes are unclear. While these microvilli certainly increase the surface area, Fox (1979) quotes papers showing their dissimilarity with the absorptive intestinal microvilli indicating possibly a greater role in foetal excretion than accretion, and also showing their absence over the surface of the vasculo-syncytial membranes suggesting they play no role in gas exchange. Stacey (1990), however, suggests that these areas provide specialized sites for the transport of macromolecular substances such as immunoglobulins, as this obviates the need for crossing an interstitial space.

#### Species variability.

The chorio-allantoic placenta shows more variability among species than virtually any other organ, not only in its development as already touched upon, but also in its structure. Thus the rabbit, rat, and guinea pig placenta have their foetal blood vessels interconnected to form a complex capillary network, among which maternal blood spaces

intertwine, the labyrinthine arrangement, in contrast to the villous arrangement of the human and sheep placenta. The number of tissue layers between the maternal and foetal blood varies between the species, varying from five in the sheep to three in the human. Even the cytotrophoblast may be present as a single, double, or triple layer depending on the species. The structural variation of some animal placentas' is listed in table 1 indicating the structural diversity despite a common function.

Grosser (1927) attempted to classify placentas on the basis of the number of tissue layers between maternal and foetal blood with the prediction of an inverse correlation between placental permeability and the number of its component tissue layers and thickness. Grosser's scheme has however been much criticised: there is no correlation between the thickness of the placental membrane and the number of its component layers (Faber & Thornburg, 1983) and electron microscopy has shown one of Grosser's placental types to have been misclassified (Lawn et al, 1969). Nevertheless, the scheme has withstood the test of time and remains the most useful placental classification system.

#### Overview of the mechanisms of placental transfer.

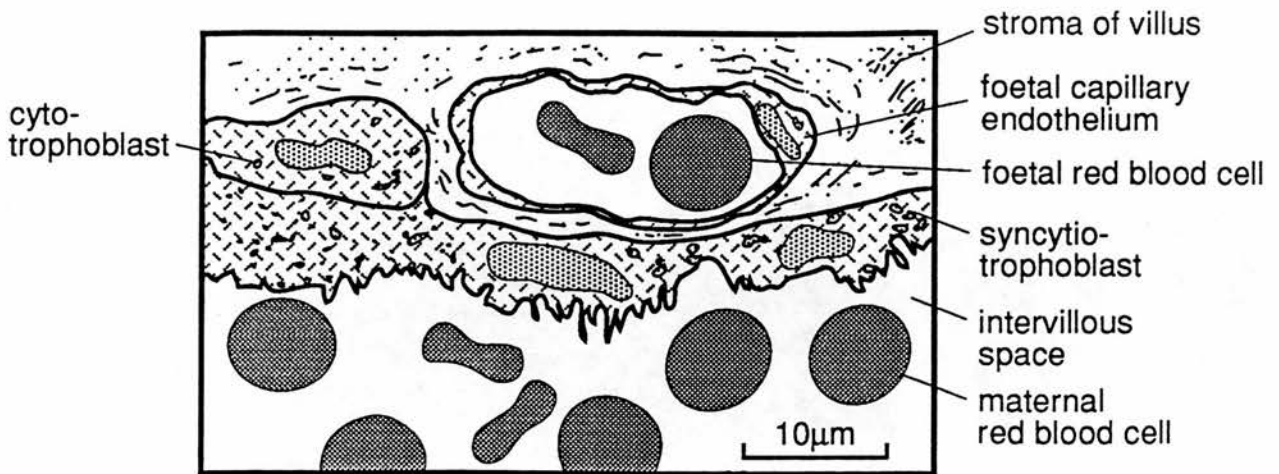
While the placental membrane functions as a single morphophysiological unit, it is infact composed of three different tissue layers, trophoblast, mesoderm, and capillary endothelium (Fig 2a). Each of these layers will

Table 1. Some species differences in placentation

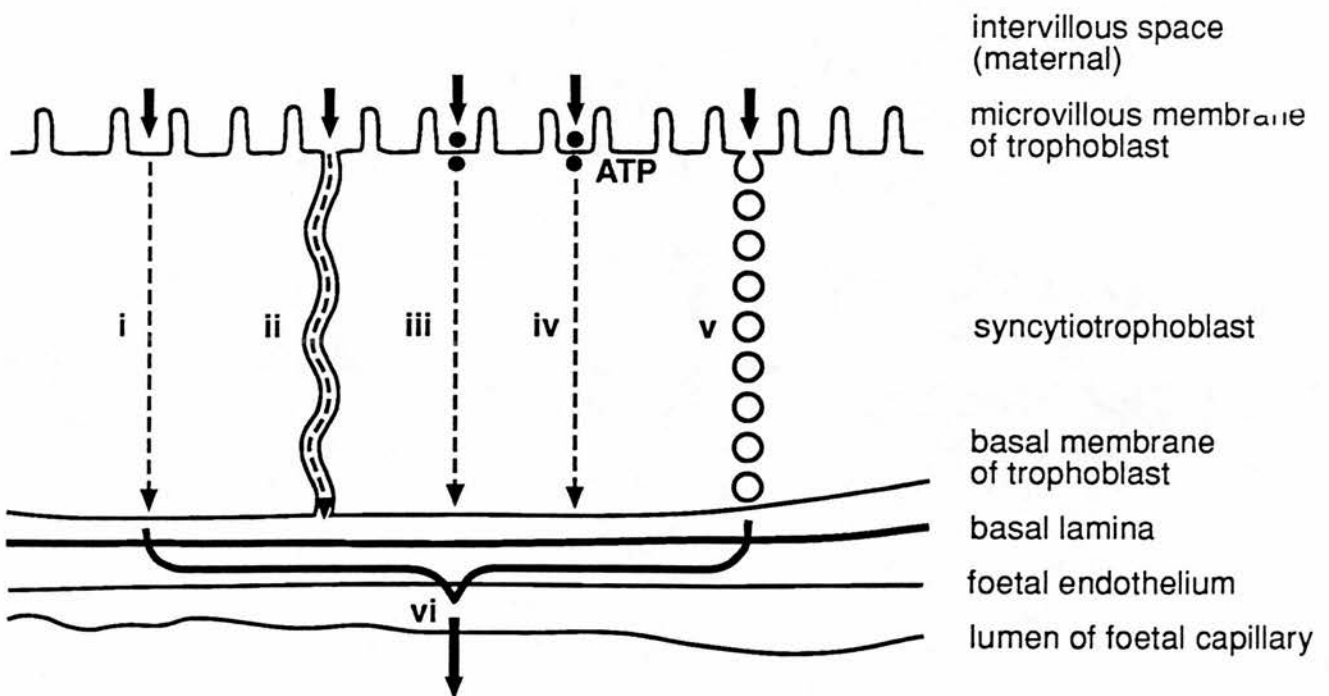
Placental classification	Placental tissue layers between maternal and foetal blood <sup>1</sup>	Species	Gross shape	Vascular arrangement
epitheliochorial	MCE-MCT UL TR-FCT-FCE	sheep	cotyledonary	villus
endotheliochorial	MCE-----TR-FCT-FCE	dog	zonary/discoid	labrythine
haemochorial	TR(x2)-FCT-FCE	rabbit	discoid	labrythine
haemochorial	TR(x1)-FCT-FCE	human	discoid	villus
haemochorial	TR(x3)-FCT-FCE	mouse/rat	discoid	labrythine

<sup>1</sup>adapted from Faber & Thornburg (1983)

MCE., maternal capillary endothelium; MCT., maternal connective tissue; UL., uterine lumen; TR., trophoblast (x number of layers); FCT., foetal connective tissue; FCE., foetal capillary endothelium



a) The placental membrane



b) Transport mechanisms across the placental membrane (after Morris and Boyd, 1988)  
 i) diffusion of lipophilic substances, ii) diffusion of hydrophilic substances,  
 iii) facilitated diffusion, iv) active transport, v) transcytosis, vi) transcapillary mechanisms.

Fig 2. The placental barrier and its transport mechanisms

not necessarily have the same permeability characteristics and transport mechanisms, and indeed are likely to be quite dissimilar.

a) Trophoblast. The syncytiotrophoblast is a syncytium with few if any mechanisms for paracellular transport while the cytotrophoblast is a discontinuous layer.

b) Mesoderm. This is absent in the vasculo-syncytial membrane where it is represented merely by a basement membrane which is a continuous feltwork around the foetal capillaries composed of glycoproteins, collagen and other fibrillar substances that is likely to act as a size and charge barrier.

c) Capillary endothelium. The foetal capillary endothelium has intercellular junctions but unlike visceral capillary endothelium which has transcellular circular openings called fenestrae, electron microscopy has shown the foetal capillary endothelium to be of the continuous type, lacking these transcellular openings. The intercellular junctions in the foetal capillary will allow paracellular as well as transcellular mechanisms of transport compared to the transcellular routes that are the only means of transfer across the syncytiotrophoblast.

Transfer across those of the above tissue layers which are entirely cellular will involve transfer across cell membranes. Membrane structure is therefore a major influence on the mechanisms of placental transfer. Cell membranes in the placenta, like cell membranes elsewhere,

are constructed of a bilayer of amphipathic lipid made up of phospholipids, glycolipids and cholesterol. A diagrammatic representation of a lipid bilayer is shown in Figure 3b).

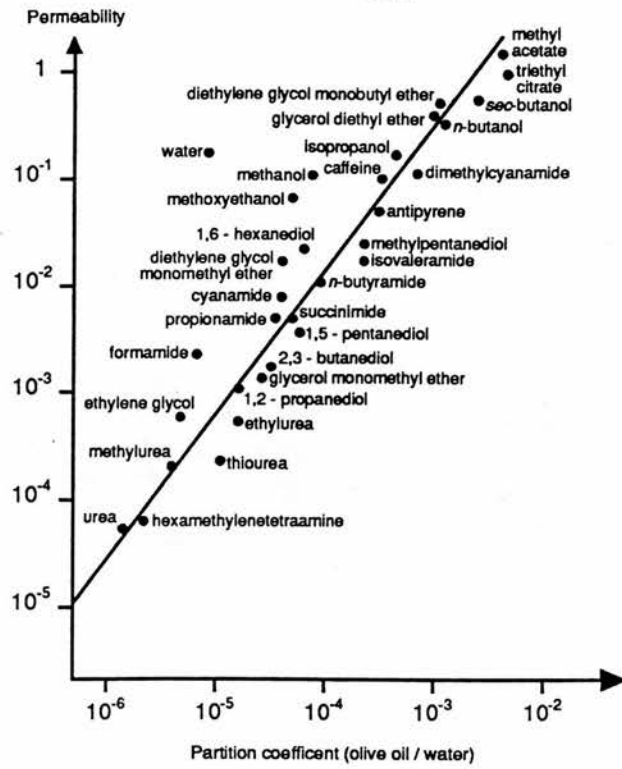
All cell membranes contain protein, for example membrane receptors and transport proteins, and many of the proteins are infact glycosylated. At 37°C the membrane lipids are in a fluid state and this allows both lipid and protein to diffuse laterally in the bilayer, the proteins to undergo conformational change, and endocytosis and exocytosis to occur. While proteins are dispersed in the membrane in five possible ways, membrane transport carriers are likely to span the width of the membrane, Figure 3c).

#### Specific mechanisms of placental transfer (Fig 2b).

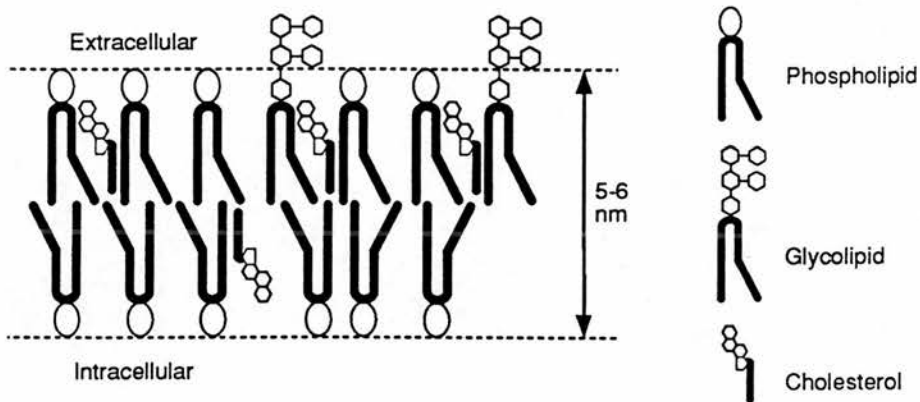
##### (a) Transcellular

The term transcellular is applied when the transfer occurs across cells and in the case of the placenta is applicable to the foetal capillary endothelium and syncytiotrophoblast. Transcellular transfer involves four main mechanisms:

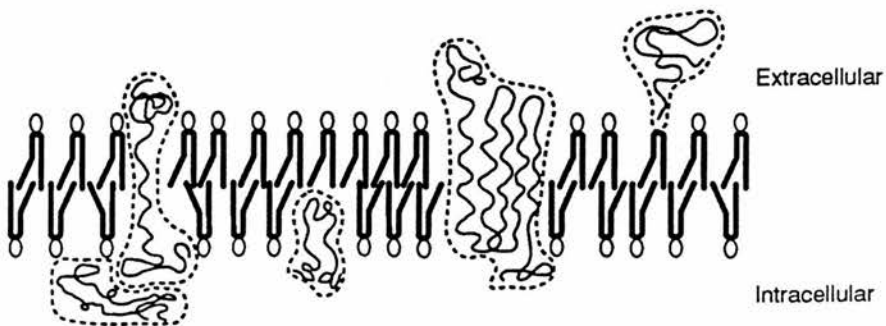
(i) Lipophilic. Lipid soluble substances for example the respiratory gases, unconjugated bilirubin, and alcohol cross the placenta readily. This is explained by the ease with which lipophilic substances dissolve in the lipid bilayer structure of the cell membrane. This is evidenced by the correlation between lipid solubility as measured by partition coefficient (olive oil/water) and the substance's permeability of the plasma membrane, Figure 3a).



a) Relationship between the permeability of several non-electrolytes towards cells of the giant algae *Nitella mucronata* and their relative oil solubility. (Redrawn from Collander, 1954)



ii) Arrangement of membrane lipids in the bilayer of a plasma membrane (after Evans and Graham, 1989)



c) Distribution of membrane proteins within the lipid bilayer (after Evans and Graham, 1989)

Fig 3. Permeability and structure of the cell membrane



In the human placenta permeability for carbon monoxide is  $295 \times 10^3 \text{ ml.min}^{-1}$ , compared with a maternal uterine blood flow of  $500 \text{ ml.min}^{-1}$ , foetal umbilical blood flow of  $350 \text{ ml.min}^{-1}$ , and permeability for  $\text{Na}^+$  of  $20 \text{ ml.min}^{-1}$  (data in Faber & Thornburg, 1983) demonstrating the high permeability to lipids.

(ii) Hydrophilic transfer. Hydrophilic substances will only slowly, if at all, dissolve in the lipid bilayer so that there must be a different mechanism of transfer from lipophilic transfer. Inert hydrophilic molecules, with a distribution limited to the extracellular space, do cross the placenta and their permeability is approximately proportional to their diffusion coefficients in water. This has been taken as evidence of transfer across the placental membrane by aqueous channels or pores. In the case of the syncytiotrophoblast these pores would have to be transcellular. There have been many efforts to visualize these channels or pores by electron microscopy but all to date have been unsuccessful (Stulc, 1989). It has indeed been questioned whether such water-filled pores need to exist as distinct entities as some of their postulated dimensions are only about twice those of a water molecule. The permeability data more precisely refers to 'notional' pores which may be just areas of free volume created by thermal fluctuation of the lipid hydrocarbon chain or spaces between the highly oriented hydrocarbon tails of the quasi-crystalline array of membrane lipids into which water partitions (Reeves & Dowben, 1970, Bittman & Blau, 1972), or



actual water-filled channels, formed either as transient pores in the lipid membrane, or 'fixed' and consisting of channels lined by a three-dimensional meshwork of glycocalyx fibres creating a molecular size-selective filter or two dimensional cylindrical apertures in the cell membrane (Michel, 1980). While the permeability data for the sheep are compatible with a 'notional' pore of radius 0.4 - 0.5 nm (Boyd, Haworth, Stacey, et al, 1976) the data for the rabbit (Stulc, Friedrich, & Jiricka, 1969), guinea pig (Hedley & Bradbury, 1980) and rat (Robinson, Atkinson, Jones, et al, 1988; Stulc, 1989) indicate a non-uniform radius for the pores, some of which would have to be between ten to thirty times wider than those measured in the sheep.

(iii) Carrier mediated transfer. This refers to transfer mediated by a specific carrier mechanism usually a protein. It is stereospecific and saturable and is of two types, facilitated and active.

Facilitated transfer operates down an electrochemical potential gradient and moves substances at a faster rate than would have occurred by simple diffusion. It does not require the expenditure of cellular energy.

Active transfer is similar to facilitated transfer but transfers against an electrochemical potential and is an energy requiring process. There is much 'in-vivo' (largely in animals) and 'in-vitro' evidence that glucose crosses the placenta by facilitated transfer and 'in-vitro' evidence only that lactate does likewise. Foetal accumulation of amino acids, some vitamins, and ions such as calcium and

phosphate, against a concentration gradient is 'in-vivo' evidence of active transport that can be supported by 'in-vitro' studies.

(iv) Transcytosis and endocytosis.

Transcytosis refers to transfer across the cell by membrane-bound vesicles and endocytosis (receptor mediated or fluid phase) if the vesicles are destined for consumption within the cell itself. Transcytosis has been most clearly established in the case of the yolk sac placenta of lower animals where it accounts for all the transplacental transfer of IgG during the latter stages of pregnancy in the guinea pig. In the human it is believed to underlie the placental transfer of IgG that is quantitatively most significant in the third trimester. It is likely to be receptor mediated as there are Fc receptors on the trophoblast (Johnson & Brown, 1981) and endothelium from villous stem vessels (Matre & Johnson, 1977).

(b) Paracellular

This pattern of transfer in the placenta is only present in the capillary endothelium. The ultrastructure of human placental capillary endothelium has been less extensively studied than in many other animals. The endothelium is of the continuous variety lacking the significant number of fenestrations which are found in other capillary beds, for example pancreas, jejunum, and kidney and contains intercellular junctions that are similar in structure to those described in the guinea pig placenta which are penetrable by molecules up to 6 nm in diameter (Firth,

Bauman, & Sibley, 1983); the permeability of these latter junctions in the human has not been similarly systematically studied. Physiologically important molecules above 6 nm in diameter which would be excluded by these junctions include albumin (7 nm approx), and most other plasma proteins.

(c) Other relevant factors affecting placental transfer.

(i). Solvent drag. This refers to the enhanced transfer of solute that occurs with water movements. It has been little studied in the human placenta but as the net foetal accumulation of water at term is of the order of  $25 \text{ ml.day}^{-1}$  (Hyttén, 1979), the effect of solvent drag might be expected to be minimal. It certainly appears to be unimportant in the case of sodium transfer across the sheep placenta (Weedon, Stacey, Ward, & Boyd, 1978)

(ii). Electrical effects. In the human the maternal-foetal potential difference is small in the pre-term infant at  $-2.7 \text{ mV}$ , and insignificantly different from zero at term (Morriss & Boyd, 1988). While the ability of the placental membrane to discriminate against the charge on molecules has not been investigated in the human, such charge discrimination has been raised as a distinct possibility in the guinea pig placenta which shares with the human a haemomonochorial structure (Hedley & Bradbury, 1980).

'In-vivo' animal studies of placental permeability.

'In-vivo' animal studies suggest that placentas may be divided into two groups on the basis of their passive

permeability characteristics; those like the sheep which present a tight barrier barely permeable to CrEDTA and impermeable to inulin and with permeability decreasing rapidly with increasing molecular size; and the others like the guinea pig, rat, and rabbit which are much more permeable to the larger molecules and whose permeability decreases much less with increasing molecular size (Faber & Thornburg, 1983). The values for placental permeability in these species are listed in Table 2.

(i) Placental permeability in the sheep.

The main investigations in this animal were performed by the group at University College Hospital, London and reported by Boyd et al (1976). These authors studied a chronically catheterized sheep preparation with a bolus or infusion of permeant into either the foetal or maternal circulation. The permeants used were  $^{14}\text{C}$  urea,  $^{14}\text{C}$  erythritol, and  $^3\text{H}$  mannitol, and their concentration in the opposing circulation was measured. By measuring the uterine blood flow, the uterine arterio-venous tracer concentration difference, and the transplacental concentration gradient, a placental clearance for the tracers in  $\text{ml}\cdot\text{min}^{-1}$  was calculated.

(ii) Placental permeability in the rabbit.

This work was reported by Stulc, Friedrich, and Jiricka (1969). Like the work on the sheep it used a catheterized animal preparation but acutely, not chronically, catheterized and just as importantly, the foetal side was artificially perfused at a predetermined rate with exogenous

Table 2. 'In-vitro' placental permeability for the rabbit, guinea pig, sheep and rat in the last one-third of gestation

Permeant	Mol.wt.	Placental permeability (ml.sec.g <sup>-1</sup> )				
		Rabbit <sup>1</sup>	Guinea Pig <sup>2,3</sup>	Sheep <sup>4</sup>	Rat <sup>5</sup>	Rat <sup>6</sup>
Urea	60	$1.5 \times 10^{-3}$	$1.2 \times 10^{-3}$	$2.3 \times 10^{-3}$	-	-
Erythritol	122	$7 \times 10^{-4}$	-	$1.5 \times 10^{-4}$	-	-
Mannitol	182	$12.5 \times 10^{-4}$	$1.5 \times 10^{-4}$	$7.7 \times 10^{-6}$	$7.4 \times 10^{-5}$	$9 \times 10^{-5}$
Sucrose	342	$4 \times 10^{-4}$	-	$7.4 \times 10^{-6}$	$7.2 \times 10^{-5}$	$6.2 \times 10^{-5}$
CrEDTA	387	-	-	$4.5 \times 10^{-5}$	$2 \times 10^{-7}$	$5.2 \times 10^{-5}$
FTIC-dextran 3000	3000	-	-	$1.2 \times 10^{-5}$	-	-
Inulin	5200	$1.8 \times 10^{-4}$	$2.6 \times 10^{-5}$	-	$2.2 \times 10^{-5}$	$1.4 \times 10^{-5}$

<sup>1</sup>From Stulc, Friedrich, & Jiricka, (1969).

<sup>2</sup>From Thornburg & Faber, (1977).

<sup>3</sup>From Hedley & Bradbury, (1980).

<sup>4</sup>From Boyd, Haworth, Stacey & Wood, (1976) and an assumed placental weight of 450g.

<sup>5</sup>From Robinson, Atkinson, Jones & Sibley, (1988).

<sup>6</sup>From Stulc & Stulcova, (1986).

Placental weight was assumed to be 4g and 5g respectively for the rabbit and guinea pig (Dawes, 1968) and 450g for the sheep assuming a fetal/placental weight ratio of 8:1 (Faber & Thornburg, 1983).

donor blood. A placental clearance was derived as in the sheep.

(iii) Placental permeability in the guinea pig.

There have been two reported studies of 'in-vivo' placental permeability in this species, Thornburg & Faber (1977) and Hedley & Bradbury (1980). The former authors used a chronically catheterized preparation but a second anaesthetic was required for the study as catheters for vascular access were inserted on the maternal side at this time (the first anaesthetic was used to ligate the vitelline vessels and so exclude the yoke sac placenta). The maternal side was perfused and a placental clearance calculated for  $^{14}\text{C}$  urea,  $^3\text{H}$  mannitol, and  $^{14}\text{C}$  inulin by measuring the total foetal uptake at set times. Hedley & Bradbury (1980) used an acutely prepared preparation with intravenous injection of tracer to label the blood perfusing the maternal side and similarly calculate a placental clearance for  $^{14}\text{C}$  erythritol,  $^{51}\text{Cr}$  EDTA, and FITC Dextran 3000 (Table 1). These authors, however repeated the experiment but perfusing the foetal side with a physiological salt solution with the placenta still 'in-situ' within the uterus, and calculated a clearance by measuring the uptake of permeant into the fluid perfusing the umbilical circulation. This resulted in the clearance of  $^{14}\text{C}$  erythritol doubling, that for  $^{51}\text{Cr}$  EDTA trebling, and that for FITC-Dextran 3000 quadrupling compared with the intact foetal circulation.

(iv) Placental permeability in the rat.



Two studies have been undertaken on the rat with the placenta perfused 'in-vivo'. Stulc and Stulcova (1986) used an acutely catheterised preparation of the rat placenta with an artificial perfusate to measure the transfer of radiolabelled mannitol, sucrose, raffinose, inulin from the maternal to the foetal side. Robinson et al (1988) used maternal injection of radiolabelled mannitol, sucrose, CrEDTA, inulin, and albumin and subsequent foetal accumulation to measure placental permeability in the intact placenta and a preparation similar to that of Stulc and Stulcova to provide parallel information about the placenta perfused 'in-vivo'. These authors found that, unlike Hedley and Bradbury (1980), there was no significant increase in permeability with perfusion of the placenta.

Although the above 'in-vivo' animal studies of placental permeability provide comparative data for the present study, they illustrate the difficulties in choosing the most appropriate animal model for the human placenta and also the change in permeability that may arise from artificial perfusion. It does appear however, that haemochorial placentas (rabbit, guinea pig, and rat) as a group have different permeability characteristics to those of epitheliochorial placentas (sheep) indicating that structural complexity may have some functional significance (Grosser, (1927)). Nevertheless, in the search for a suitable animal model for the human placenta histological similarity cannot be assumed to imply functional similarity.

## Chapter 2.

## Passive permeability studies of the human placenta.

Introduction.

Free diffusion is the simplest form of transfer across biological membranes and a logical starting point for any study of placental transfer. The rate of free diffusion is a function of passive permeability, and measuring the rate of diffusion across the human placenta will allow the passive permeability of the placental membrane to be calculated. The passive permeability of the placental membrane is a fundamental characteristic that reveals much about its structure and function.

Most previous studies of passive permeability in the human placenta have used 'in-vitro' preparations for technical and ethical reasons. Few 'in-vivo' studies have been performed and these have used only single permeants so that different data have to be combined to construct a permeability profile.

'In-vitro' studies.

These are of two main types: intact preparations, using artificial perfusion of a part usually a lobule of the placenta, and isolated preparations, using isolated placental tissue either placental slices or placental



villous tissue, specially prepared microvillous membrane vesicles, or trophoblast cell cultures.

(i). 'In-vitro' perfusion of the human placental lobule.

This technique involves cannulating the placenta immediately after delivery to establish circulations on the foetal and maternal sides of the placenta. The foetal side is perfused through a cannulated artery and vein on the periphery of the placenta, and the maternal side perfused by two cannulae inserted into the intervillous space demarcated by perfusion of the foetal side. The maternal perfusate returns through multiple venous openings in the decidual plate and is continuously collected (Illsley, Aarnoudse, Penfold et al, 1984). Using this technique, the passive permeability of the placenta to a range of hydrophilic permeants has been measured, Table 2, p 33. Although technically difficult and requiring considerable expertise, the technique allows ready access to placental tissue and a wide range of studies to be undertaken.

There are however, several limitations of the technique. As many of the placentas studied by this technique are from vaginal deliveries there is the possibility of altered function from the stress of labour. Even though all possible measures are taken to ensure placental viability, there is still an interval between the delivery of the placenta and the establishment of perfusion, during which time the placenta may suffer metabolic damage. The perfusate has to be a physiological buffer and whether the perfusion flow and pressures exactly duplicate those found 'in-vivo' remains

uncertain. Exchange and perfusion may not be uniform throughout the placenta and this has two consequences for the technique of 'in-vitro' perfusion. Firstly, the lobule chosen for 'in-vitro' perfusion may not be representative of the placenta as a whole and secondly, 'in-vitro' perfusion may alter the distribution and balance of perfusion within the lobule leading to altered placental exchange.

(ii). Isolated placental tissue preparations.

These techniques provide information about cellular uptake which is not the same as transfer across the placental membrane. However, such studies are the only way at present that information about the individual tissue layers of the placental membrane can be obtained. Studies with directly dissected villous tissue have shown it to have a large extracellular water space that is only slowly penetrated by inulin (Smith & Depper, 1974), indicating that the trophoblast provides a major barrier to free diffusion. Studies with isolated microvillous membrane vesicles have demonstrated and localized a similar barrier to free diffusion not only to the syncytiotrophoblast plasma membrane, but to its maternally facing side (Smith, 1981; Bissonnette 1981).

'In-vivo' studies.

There have been various studies attempting to measure the 'in-vivo' permeability of the human placenta to a variety of permeants. The first systematic study was by Flexner and co-workers (Flexner, Cowie, Hellman, et al, 1948) who measured

the placental transfer of  $^{24}\text{[Na]}$  at term and the end of the first trimester. The authors used a maternal injection of radiolabelled sodium, and measured its disappearance from the maternal circulation and its appearance in the foetal circulation at birth. From a volume of distribution factor for the foetal plasma concentration, a unidirectional flux for sodium was calculated, expressed as milligrams of sodium transferred per gram of placenta per hour. Sodium, however is not inert and transferred solely by passive diffusion as there exist in the placenta specific sodium transport mechanisms, for example Na - amino acid co-transport (Boyd, 1981). Studies of the placental transfer of  $^{125}\text{[I]}$  insulin (Kalhan, Schwartz, & Adam, 1974) are subject to similar criticisms as insulin is a biologically active molecule with a short half-life.

Other studies attempting to measure placental transfer have been undertaken with either tracer analogues or inert, hydrophilic, molecules. Holmberg and colleagues, (Holmberg, Kaplan, Karvonen, et al, 1956) studied the placental transfer of D-xylose, a supposedly inert aldopentose also used in gut permeability studies, and fructose. Willis et al (Willis, O'Grady, Faber et al, 1986) used cyanocobalamin, and Thornburg et al, (Thornburg, Burry, Adams et al, 1988) used inulin. The data of Holmberg et al (1956) only give maternal-foetal concentration gradients with no measure of net flux; a true permeability cannot be calculated but the data do allow the transfer of fructose and xylose to be compared with that of glucose. This comparison shows the

transfer of fructose to be one tenth that of glucose and the transfer of D-xylose to be intermediate between that of glucose and fructose which agrees with other studies of the stereospecificity of placental glucose transport (Johnson & Smith, 1980). Xylose has also been shown to be a poor marker of placental permeability in the sheep (Stacey, Weedon, Haworth et al, 1978) where its transfer was consistent with a shared transfer mechanism with D-glucose. The permeability studies with cyanocobalamin and inulin were single permeant studies which introduces further errors when trying to combine the data due to the marked individual variability in permeability that is such a feature of animal placentas.

The available data on the 'in-vivo' permeability characteristics of the human placenta are thus very limited.

Summary of background.

Exchange between the maternal and foetal vascular compartments across the placenta underlies normal foetal growth and development as it provides the necessary nutrients and removes the accumulating metabolic waste products. There are few 'in-vivo' data on placental transfer in the human because of the technical and ethical difficulties in undertaking these essentially clinical studies. Attention has therefore focused on animal and 'in-vitro' studies as a means of obtaining data on placental function which may be applicable to man. Animal studies are however of uncertain relevance to man because the known structural and functional differences between the different animal placentas may extend to the human and this has not been explored by comparative studies of animal and human placental function. 'In-vitro' studies provide data on the human placenta, but their applicability to the 'in-vivo' situation is again unproven. There are doubts about both the viability of the placenta when perfused 'in-vitro' and the validity of the results obtained by this technique. Placental tissue preparations give information about transport mechanisms but no information about quantitative foetal transfer; there is also no proof that the transfer mechanisms demonstrated 'in-vitro' have necessarily the same function 'in-vivo'.

The few 'in-vivo' studies that have been performed have analyzed the data slightly differently (see discussion) and only one permeant has been used at a time. The data are not

strictly comparable and the large inter-placental variation will further limit the sensitivity of the combined data.

Measurements of placental transfer are thus required for understanding normal placental function and as a prerequisite for demonstrating abnormal transfer. Hydrophilic transfer (passive diffusion) is the pattern of transfer examined in this study. It is: the simplest form of transfer across the placental membrane; a fundamental characteristic of the placental membrane; the starting point for any study of membrane transfer characteristics; and one of the mechanisms of placental transfer available for all smaller, hydrophilic molecules such as the inert permeants used in this study, and molecules of biochemical and nutritional importance such as D-glucose, creatinine, urea, amino acids, and thyroxine.

Aims of the research.

1. To measure the placental permeability of the human placenta 'in-vivo' to a range of inert, hydrophilic, molecular permeants that will allow the construction of a permeability profile. This will provide a measurement of placental exchange available to the foetus through passive diffusion.

2. To analyze the data in terms of one of the theories of membrane transfer, namely pore theory, to ascertain whether the data from the human placenta are consistent with this theory.

3. To provide 'in-vivo' permeability data that can be directly compared with 'in-vitro' placental perfusion data (DK Copas & MJ Landon) obtained in the same group of placentas.

4. To provide 'in-vivo' permeability data from the human that can be compared with similar data from other animal species that will help in the identification of suitable animal models for human placental function.

5. To provide measurements of placental permeability that can be compared with similar measurements in pregnancies where there is disordered foetal growth, and foetal transfer might be expected to be abnormal, for example idiopathic foetal intrauterine growth retardation or hypertensive disease of pregnancy.



## Chapter 3.

## Methodology.

Clinical.

All the studies were performed in pregnancies at term which were scheduled for delivery by elective caesarian section for reasons other than foetal or placental abnormality, for example breach presentation, unstable lie, or previous caesarian section. Such women were identified from the labour ward work book a few days before the scheduled day of delivery. On the day of admission, that is the day before delivery, both the prospective mother and father were approached, the nature of the project explained, and consent sought from both. Only when this was given was the study undertaken. Pregnancies were not studied when there was any risk of foetal abnormality or when the nursing staff were concerned about the mother's psychological state. Approximately half of the couples approached agreed to participate.

The infusate was prepared on the morning of delivery. For the studies using only mannitol and inulin the infusate was prepared on the ward just before clinical use; for the studies with all four permeants, mannitol, lactulose, CrEDTA, and inulin, the infusate was prepared for clinical use by the pharmacy department that morning. About one hour

before the scheduled time of caesarian section a large bore, 14 G cannula was inserted into a maternal arm vein. A large bore cannula was standard anaesthetic practice to permit the administration fluids and drugs and in case large amounts of fluid had to be given rapidly. The pre-infusion blood sample was withdrawn through the cannula while all subsequent blood samples were obtained by a small butterfly needle inserted into a contralateral arm vein; deadspace was withdrawn and patency maintained with heparinized saline. A maternal urine sample was obtained at the time of urethral catheterization which was done routinely for caesarian section and was performed before the infusion was commenced. A bolus of the infusate was followed by a continuous infusion until the time of delivery. The two infusates were given at a different rate to keep the amount of administered mannitol and inulin approximately the same despite the different composition of the two infusates. The two infusion regimes are listed in Table 3.

Table 3. The two different infusion regimes used.

Infusate composition	Volume of bolus	Rate of continuous infusion
Mannitol/ inulin	100 ml	60 ml.hr <sup>-1</sup>
Mannitol/inulin/ CrEDTA/lactulose	165 ml	99 ml.hr <sup>-1</sup>

Permeant composition of each regime is detailed in materials section, page 86.

The infusion rate was controlled with an IVAC infusion pump, model 531, and the bolus given as free flow under gravity, approximately 10 minutes for mannitol and inulin, and 15 minutes for the solution with the four probes. Maternal blood samples were taken at approximately 20 minute intervals during the infusion. The exact length of the infusion was determined by the schedule on the delivery suite.

At delivery a sample of venous cord blood and if possible amniotic fluid was obtained. Neonatal urine was collected into a small plastic urine bag glued onto the perineum with medical grade adhesive, Dow Corning 355 Medical Grade Adhesive. The urine bag was continuously aspirated with a vacuum pressure of 40cm water (plate 2). This allowed the infant a normal amount of freedom and the mother to provide as much care for the infant as she wanted in the first couple of days, bearing in mind that the mother has just had a caesarian section with a midline abdominal incision. The urine collection was maintained for between 50 to 60 hours and the urine was collected as separate timed collections.

The urine was either analysed straight away, or more usually, frozen for analysis later. The blood was allowed to separate and the serum and amniotic fluid frozen until analysed.

The protocol and procedures were approved by the hospital/clinical research centre ethical committee.

### Permeants.

Four permeants were used (Fig 4).

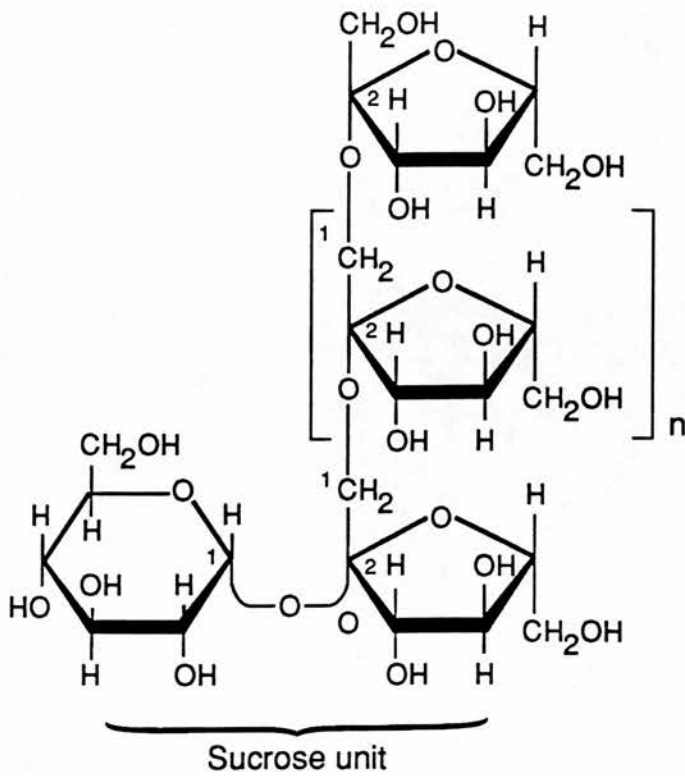
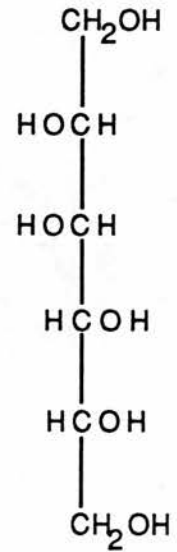
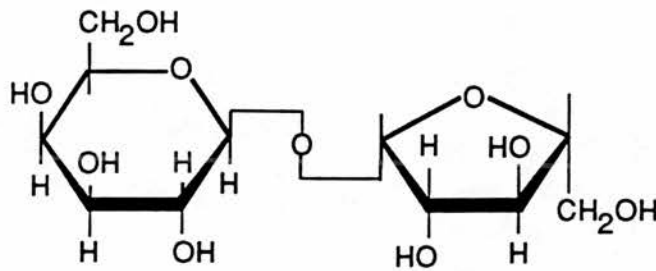
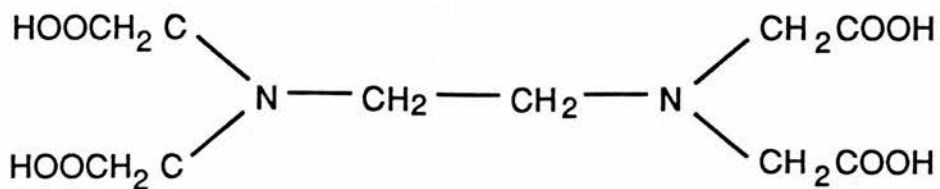
#### (i) Inulin.

Inulin is a linear fructose polymer with one terminal glucose residue which is obtained from the tubers of *Dahlia variabilis*, *Helianthus tuberosus*, and other genera of the family Compositae. The average molecular weight is 5000-5500 daltons, indicating approximately 30 sugar residues per inulin molecule. Molecular radius has been calculated at approximately 1.4nm (from sources in Normand, Olver, Reynolds and Strang 1971). It is an inert, non-metabolizable and hydrophilic molecule that has long been used as a marker for the extracellular space or the measurement of glomerular filtration rate (GFR). Continuous infusion of inulin has been used safely to measure maternal GFR in pregnancy (Davison & Hytten 1974).

Brandes, Abramovici & Peretz (1971) were the first to show that inulin given intravenously to the mother, passed across the placenta into foetal venous blood and urine, and entered amniotic fluid in measureable amounts. Other studies by the same group (Brandes, Abramovici & Reich 1970) using paper chromatography showed that the inulin transferred across the placenta remained as a polymer but as it was compared with the monosaccharides fructose, glucose, and raffinose, and remained at the origin of the chromatogram, a change in the molecular size distribution was not excluded.

#### (ii) CrEDTA.

This is a molecular complex of ethylenediaminetetraacetic

**INULIN****MANNITOL****LACTULOSE****EDTA**

Pentadentate bonding  
of chromium to EDTA

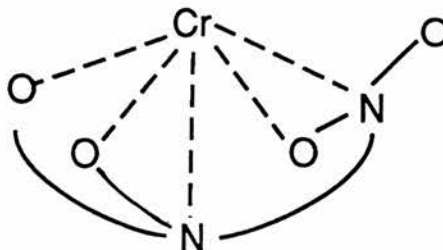


Fig 4. Placental permeants used in the current study

acid (EDTA) with Cr(III). EDTA chelates a wide spectrum of metals with a high formation constant that ensures the metal ion remains firmly bound in the complex. CrEDTA is: physiologically inert, not bound by plasma proteins, does not penetrate red blood cells, is not taken up or metabolized by any organ other than the kidneys which filter it in a manner virtually identical to that of inulin, and in adults 98% of an administered dose is excreted by the kidneys within 24 hours (Amersham data sheet). The use of  $^{51}\text{[Cr]}$  EDTA to measure GFR was first reported in 1966 and is now a standard technique in both adults and children.  $^{51}\text{[Cr]}$  EDTA has more recently (Maxton, Bjarnason, Reynolds, et al, 1986) been used as a marker in intestinal permeability studies. CrEDTA therefore is thought to be a safe permeant for studying placental permeability with the added advantage that it is not a saccharide like the other three permeants providing a form of internal control for the study.  $^{51}\text{[Cr]}$  EDTA being radioactive could not be used during pregnancy, but non-radiolabelled CrEDTA could be safely used instead with measurement through assay of the elemental chromium.

The Cr(III)EDTA complex is a complicated and controversial compound that has chromium complexed with EDTA in an equimolar ratio. The molecular weight of the complex is 387 as the disodium salt. The complex of Cr(III) with EDTA is generally accepted to be pentadentate, with the Cr bound to the EDTA complex at 5 sites. The shape of the molecule is a distorted octahedron with a surrounding hydration shell that contains up to 6 water molecules.



The standard dose of  $^{51}\text{CrEDTA}$  for GFR measurement is 40-80  $\mu\text{Ci}$  corresponding to 40 - 80  $\mu\text{g}$  of elemental chromium (specific activity of supplied  $^{51}\text{CrEDTA}$  approximately 1  $\mu\text{Ci}.\mu\text{gCr}^{-1}$ ). The normal dietary intake of elemental chromium is 60 - 100  $\mu\text{g}.\text{day}^{-1}$  and the total body content 6 mg (Venugopal & Luckey, 1978). The normal urinary concentration of elemental chromium is approximately 1  $\mu\text{g}.\text{l}^{-1}$ , and by analogy with the placental transfer of mannitol which was already known, a bolus and infusion of 100  $\mu\text{g}$  elemental chromium corresponding to 0.8 mg of CrEDTA were likely to give satisfactory foetal blood and urinary levels. This dose did not contain greater than the daily intake of elemental chromium, and the dose of EDTA was well below the upper limit of 50  $\text{mg}.\text{kg}^{-1}.\text{day}^{-1}$  recommended for its therapeutic use as a chelating agent.

(iii) Lactulose.

Lactulose (4-O- $\beta$ -D Galactopyranosyl D-fructofuranose) molecular weight 342, is a disaccharide consisting of one molecule of galactose joined to one molecule of fructose. It is inert, not metabolised within the body, and there is no evidence of endogenous production. Its volume of distribution is limited to the extracellular fluid space, and its excretion is wholly and solely by the kidney. Less than one percent of ingested lactulose is absorbed from the neonatal intestine, at a period when the intestine is especially permeable to intact sugars and proteins. After an intravenous injection 95% of the injected dose is recoverable in the urine (Menzies, 1983; Weaver, Laker, and



Nelson, 1984). Lactulose is infact present in some 'ready to feed' liquid infant milks. It is formed as result of the terminal sterilisation used in the preparation of these feeds which converts up to 6% of the lactose into lactulose (Beach, and Menzies, 1984).

Lactulose is widely used as a safe osmotic laxative with a usual dose for an adult being 20 g twice daily. In hepatic encephalopathy, where lactulose has a special place in removing protein from the gut, doses of 30 g three times daily are recommended. Lactulose has been widely used as a marker to investigate intestinal permeability in adults and more recently for similar studies in the neonatal period (Weaver, Laker, and Nelson, 1984)

(iv) Mannitol.

Mannitol is a monosaccharide with molecular weight of 182. It is inert, and not metabolised within the body. Endogenous production has been suggested in adults but Weaver et al (1984) could find no evidence of this in the neonates they studied. Like lactulose, mannitol is a normal constituent of several proprietary infant milk feeds but is absent from breast milk. Its volume of distribution is the same as lactulose, namely the extracellular fluid (though it does penetrate red blood cells slowly 'in-vitro'), and its excretion is similarly by the kidney, with 95% of an intravenous dose being recoverable in the urine (Menzies, 1984). Less than 5% of orally administered mannitol is absorbed in the neonatal period.



Basso, Fernandez, Althabe, et al, (1977) were the first to show that mannitol given to the mother just before delivery passed across the placenta and could be detected in the amniotic fluid where the concentrations exceeded those in maternal blood. Levels in cord blood were not measured.

Mannitol is widely used as one of the few effective therapies for raised intracranial pressure largely outside the neonatal period, but at all ages, including the neonatal, is used as an osmotic diuretic in the therapy of renal failure. Adult doses of mannitol vary from 50 - 200 g over 24 hours; the neonatal dose is quoted as 1 - 2 g.kg<sup>-1</sup> body weight, well in excess of our measured net transfer to the foetus of 200 - 400 mg.

All four permeants are inert, hydrophilic, markers that are wholly and solely cleared by the kidney: they are believed to cross physiological membranes by passive and unmediated diffusion.

#### Analytical.

##### (i) Inulin

#### Principle.

Inulin was assayed by the method of Heyrovsky (Heyrovsky 1956). This uses an alcoholic solution of purified indol-3yl-acetic acid to identify the fructose end product of inulin hydrolysis. In the presence of fructose and concentrated hydrochloric acid this reagent gives a quantitative purple-blue colour. This reaction is claimed to

be of relatively high specificity and sensitivity with only fructose and fructose containing saccharides giving the reaction in comparable concentrations; interference by glucose is stated to be of the order of 0.5 - 1 %. As proteins in the urine do not interfere with the reaction deproteinization of this fluid is not necessary.

#### Reagents:

All reagents used for the inulin assay were obtained from BDH Ltd. Dagenham, Essex.

A 0.5g.100 ml<sup>-1</sup> solution of 4 indol-3yl-acetic acid was prepared by adding 125 mg of the pure crystalline material to 25 ml 96% ethanol alcohol (Analar) and vortex mixed until fully dissolved. This solution was kept at 4°C and remade as necessary.

Inulin standards were prepared from chemical grade inulin powder. A stock solution of 1 mg.ml<sup>-1</sup> was made by dissolving 50 mg of inulin in 20 - 25 ml distilled water, heating till dissolved, and then making up to 50 ml with distilled water. This stock solution was then diluted 1, 2, 4, 6, and 8ml to 100 ml distilled water to give the working standards 10, 20, 40, 60, and 80 µg.ml<sup>-1</sup>. Where other standards were required, for example 120 and 160 µg.ml<sup>-1</sup>, these were prepared in a similar manner from the same stock solution.

Trichloroacetic acid was prepared as a 20 g.100 ml<sup>-1</sup> solution in distilled water.

**Sample preparation:**

(1) Serum & Amniotic fluid. 250  $\mu$ l of serum or amniotic fluid was added to 1.25 ml of distilled water and 1 ml of 20% trichloroacetic acid solution and vortex mixed. The specimen was then spun for 5 minutes at 2500 rpm and the crystal clear supernatant removed for subsequent assay. Dilution of the original sample was thus 1:10.

(2) Urine & Infusate. The urine and infusate samples were diluted accordingly:

infant urine	1 in
	10 (0.5ml to 4.5ml dist. H <sub>2</sub> O)
maternal urine	1
	in 250 (400 $\mu$ l to 100 ml dist H <sub>2</sub> O)
infusate	1 in 1000
	(250 $\mu$ l to 250ml dist H <sub>2</sub> O)

The dilutions of the urine specimens were performed as soon as possible after the urine was collected to minimize the precipitation of inulin (Robertson, Hytten, & Cheyne 1970) and to bring the inulin concentration within the range of the prepared standards.

**Assay technique:**

To 0.5 ml of the diluted urine, serum or amniotic filtrate, blank (distilled water) and standards, 100  $\mu$ l of indol-3yl-acetic acid solution

and 4 ml of concentrated hydrochloric acid (SG 1.18 Analar) was added and vortex mixed. This mixture was incubated in a water bath in screw capped glass tubes at 37°C for 75 minutes, cooled in cold tap water for a further 30 minutes and then read at 530 nm against a water blank. Each assay was performed in duplicate and the mean of the two results taken.

The sample value was read off against the standards run with each assay (Fig 5) and the inulin concentration in the original sample determined by multiplying this value by the dilution made. The accuracy of the assay was checked in two ways.

(1) The intra-assay variability was assessed by multiple assays of the same sample; 12 determinations gave a range of 53 - 59  $\mu\text{g}.\text{ml}^{-1}$ , mean 56 and standard error of the mean 0.6

(2) As inulin is extremely stable the same standards were able to be used for up to 4 months. When a new set of standards was made up it was checked against the old one. The difference between the two was always less than 5%.

#### Column Chromatography.

Column chromatography using Sephadex gel filtration columns was used firstly, to confirm the identity of the inulin being measured and secondly, to separate the inulin from interfering lower molecular weight substances when quantifying the neonatal urinary inulin excretion. In both

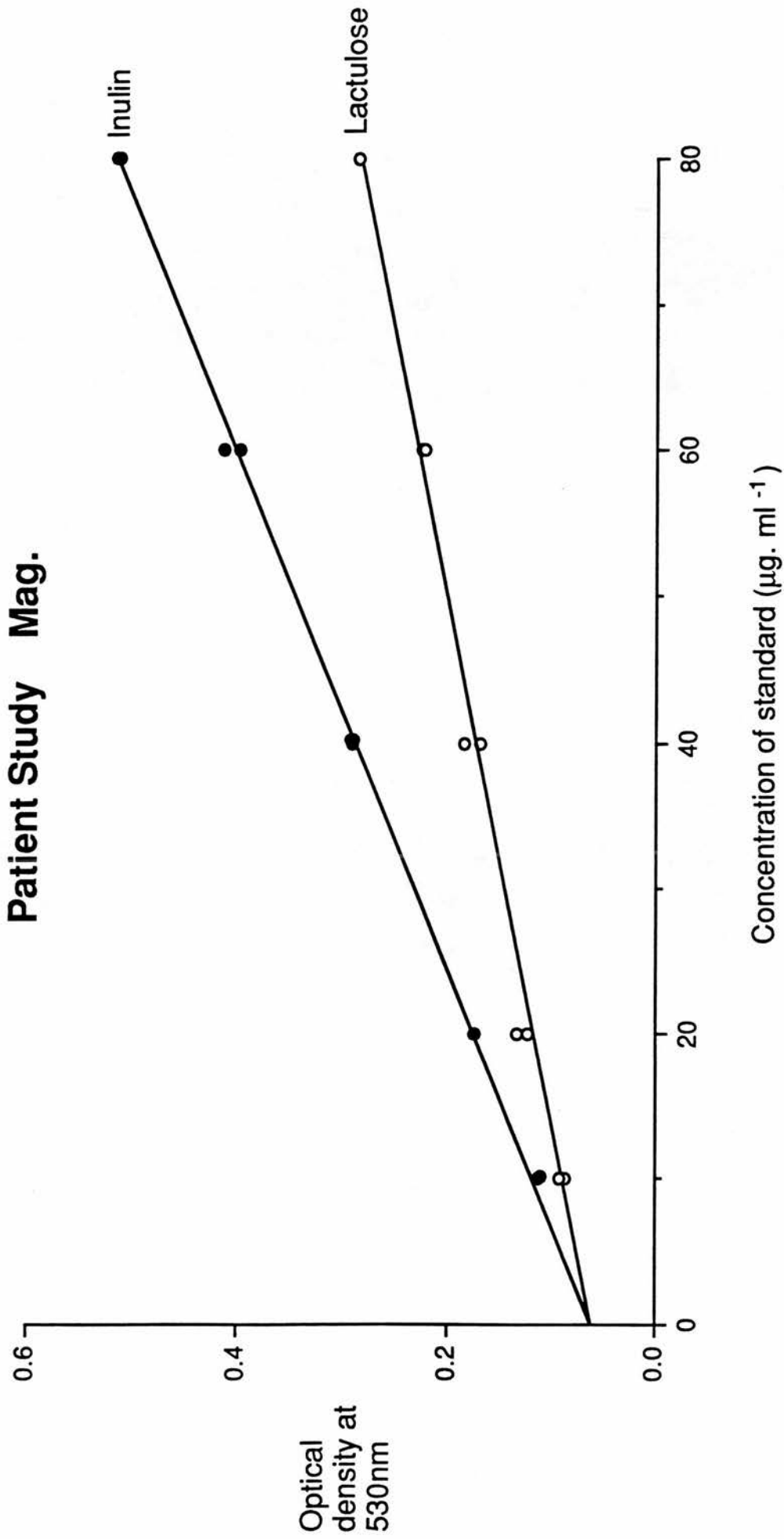


Fig 5. Standard curve of Heyrovsky reaction with inulin and lactulose standards

cases the same basic technique was used though the grade of Sephadex and the length of column varied. The eluent was 0.1 mmolar phosphate buffered saline, PBS, (exact composition NaCl 9.3 mM, KCl 0.2 mM, NaHPO<sub>4</sub> 0.05 mM, and KH<sub>2</sub>PO<sub>4</sub> 0.1 mM ) and 0.02 g.dl<sup>-1</sup> azide was added to prevent bacterial growth. Potassium dichromate and blue dextran were used to characterise the columns. The sample volume was less than 0.5 ml for the 15 cm column and 1 ml for the 40 cm column. The elution rate was 2 ml.hr<sup>-1</sup> and the fraction volume 600 µl. <sup>3</sup>[H] inulin (Amersham International) was used as internal standard (approximately 0.4 µCi) and was measured by scintillation counting on a LKB 1215 Rackbeta. The samples were prepared by adding 4 ml of scintillant to 100 µl of the eluent. Limits for counting were set at 100 seconds and 10 K.

#### Identity of Excreted Inulin.

Gel filtration chromatography on Sephadex G-50 confirmed that the infused inulin was not significantly changed by passage through the placenta into foetal fluids (Fig 6). Sephadex G-50 was chosen as inulin lay within its range of separation of polysaccharides (Pharmacia, 1985). The molecular size distribution of infused inulin was compared with that appearing in the foetal and maternal urine. A 15 x 1 cm column was used. Inulin is a polydisperse molecule and the prolonged tail on the chromatogram is due to lower molecular weight forms. The <sup>3</sup>[H] inulin is also shown to be of slightly lower molecular weight than the chemical inulin;



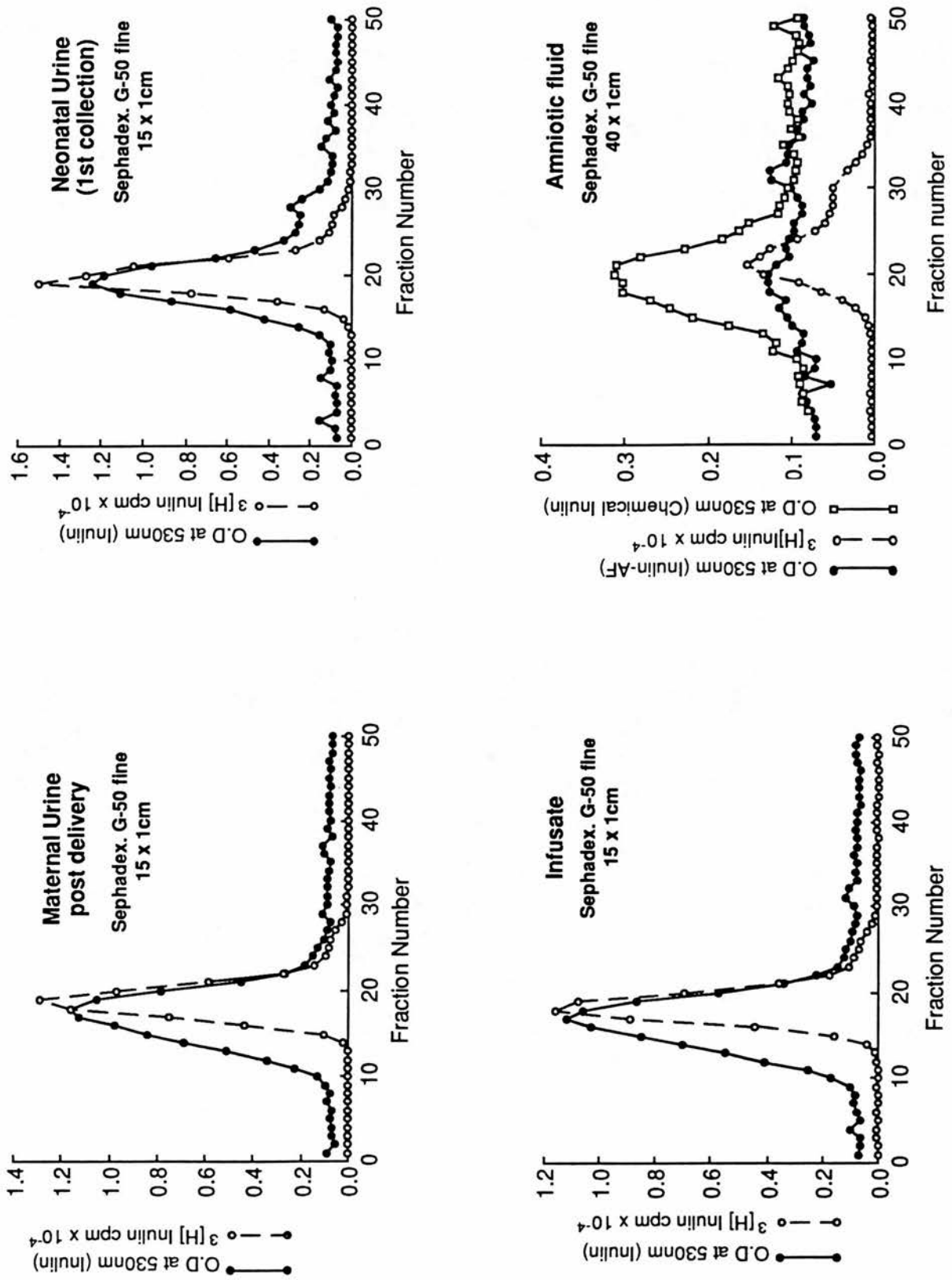


Fig 6. Column chromatography of infused and excreted inulin

this is also the case for  $^{14}\text{C}$  inulin (Coulthard & Ruddock, 1983). Amniotic fluid was analysed separately on a 40 x 1 cm column. The concentration of inulin was much lower in the amniotic fluid and in all cases in which sufficient amniotic fluid was available for analysis there was chromatographically identifiable inulin present, but the peak of inulin was sometimes barely above background. In Fig 6 the profile of chemically pure inulin has been superimposed to help identify the inulin in amniotic fluid.

When there was insufficient inulin in the sample to be applied to the column to give a good sized peak it was necessary to concentrate the sample by freeze drying. This was the case for all the samples of amniotic fluid and the most dilute of the urine collections. The sample was first shell-frozen, and then dried at  $-10^{\circ}\text{C}$  with a vacuum of 0.5 Torr ( $\sim 67 \text{ Nm}^{-2}$ ). The recoveries on 2 samples after freeze drying were 90 and 110%.

#### Measurement of Inulin in Body Fluids.

In the body fluids under study there were substances that cross-reacted in the assay for inulin that were not inulin. These were allowed for in different ways depending on whether the body fluid was either serum, or urine and amniotic fluid. In the second group of patient-studies where lactulose was infused, the lactulose cross-reacted with the inulin because of its fructose component. This was allowed for in sera or urine in a similar manner to that of the cross-reacting material.

(1) Serum. In the patient-studies in group 1 where only mannitol and inulin were infused, serum measurements of inulin were performed as above. In the patient-studies in group 2, where the Heyrovsky reaction measured both the inulin and lactulose present, inulin could not be measured directly. The concentration of lactulose was first measured by gas chromatography and converted to an optical density from a calibration curve constructed from a range of lactulose standards assayed as for inulin and run with each assay. The calculated optical density for the measured lactulose concentration was then subtracted from the optical density measurement of the sample to obtain the optical density due to inulin itself and this was read off the inulin standard curve as previously to give the true inulin value.

(2) Urine and amniotic fluid. The blank value for neonatal urine in the inulin assay varied between neonates and the time after delivery (figs 7 & 8), rendering invalid the simple subtraction of a single value for background that would apply to all patient-studies. Gel filtration chromatography using Sephadex G-50 was therefore used to separate the inulin from the background activity and quantify it.

Column chromatography was carried out with the standard technique outlined previously. A representative sample, usually 10 ml, of the total neonatal urinary excretion was prepared by taking proportionate amounts of each individual urine collection and pooling them. A variable amount of this

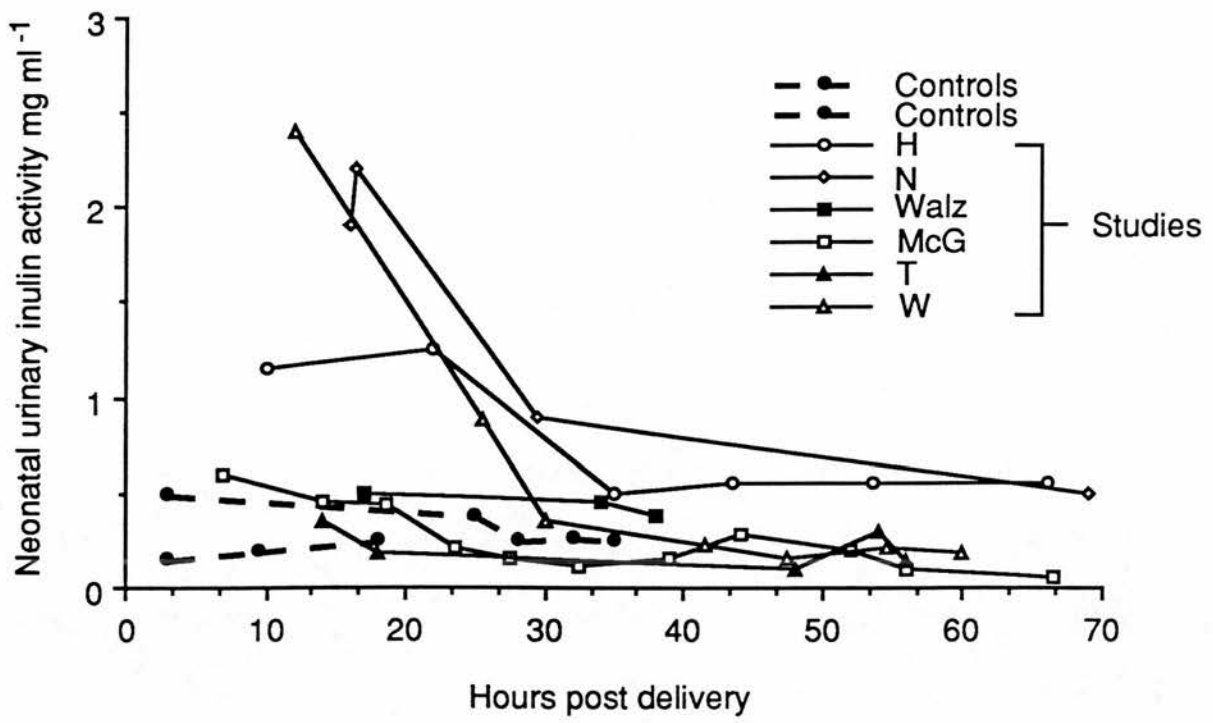


Fig 7. Neonatal urinary excretion of inulin activity (Heyrovsky reaction) in 2 control infants and 6 patient-studies

Study Kar

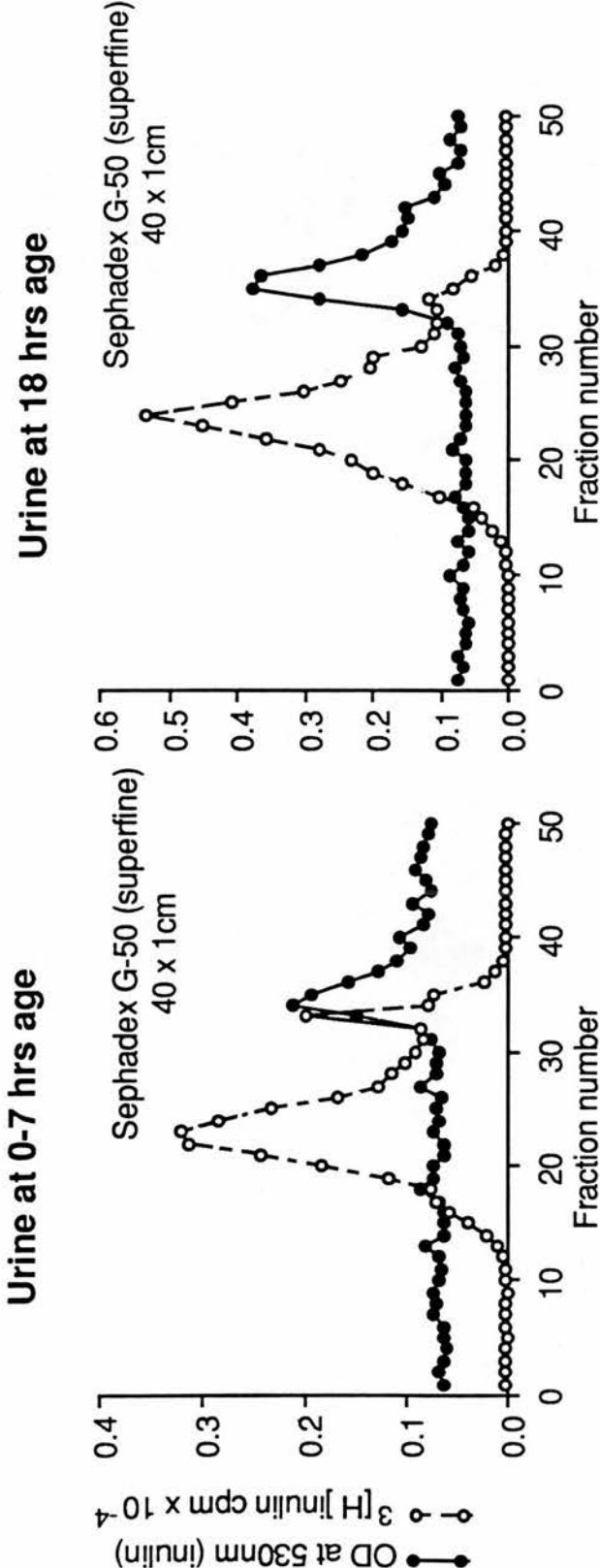


Fig 8. Column chromatography of neonatal urine from a control study

sample was applied to a Sephadex column to achieve a large enough peak of inulin activity such that the highest value was still within the range of the standard curve. Where the urine volume was large it was sometimes necessary to concentrate the representative urine sample by freeze drying a measured volume and resuspending it in a smaller volume. Out of each 600  $\mu$ l aliquot collected, 500  $\mu$ l was assayed by the method of Heyrovsky for inulin and 100  $\mu$ l scintillation counted for  $^3\text{[H]}$  inulin. In the first group of patients studied, a 40 x 1 cm column of Sephadex G-50 (fine) produced satisfactory separation from the cross-reacting material (fig 9).

In the second group of patient-studies where lactulose was infused a Sephadex G-25 (superfine) (40 x 1 column) was necessary for adequate separation of the larger sized inulin from the smaller sized cross-reacting lactulose which was present in a much higher concentration (Fig 10). On the Sephadex G-25, inulin eluted at the void volume of the column while lactulose appeared in the middle of the elution volume coincident with the naturally occurring cross-reacting substance.

The amount of inulin in the proportionate urine sample was calculated from its peak area on the chromatogram. The optical density measurements of the inulin curve, identified by  $^3\text{[H]}$  inulin, were summated and divided by the number of fractions over which it was collected to give a mean optical density which was converted into a mean inulin concentration ( $\mu\text{g.ml}^{-1}$ ) by reading off the standard curve. This was

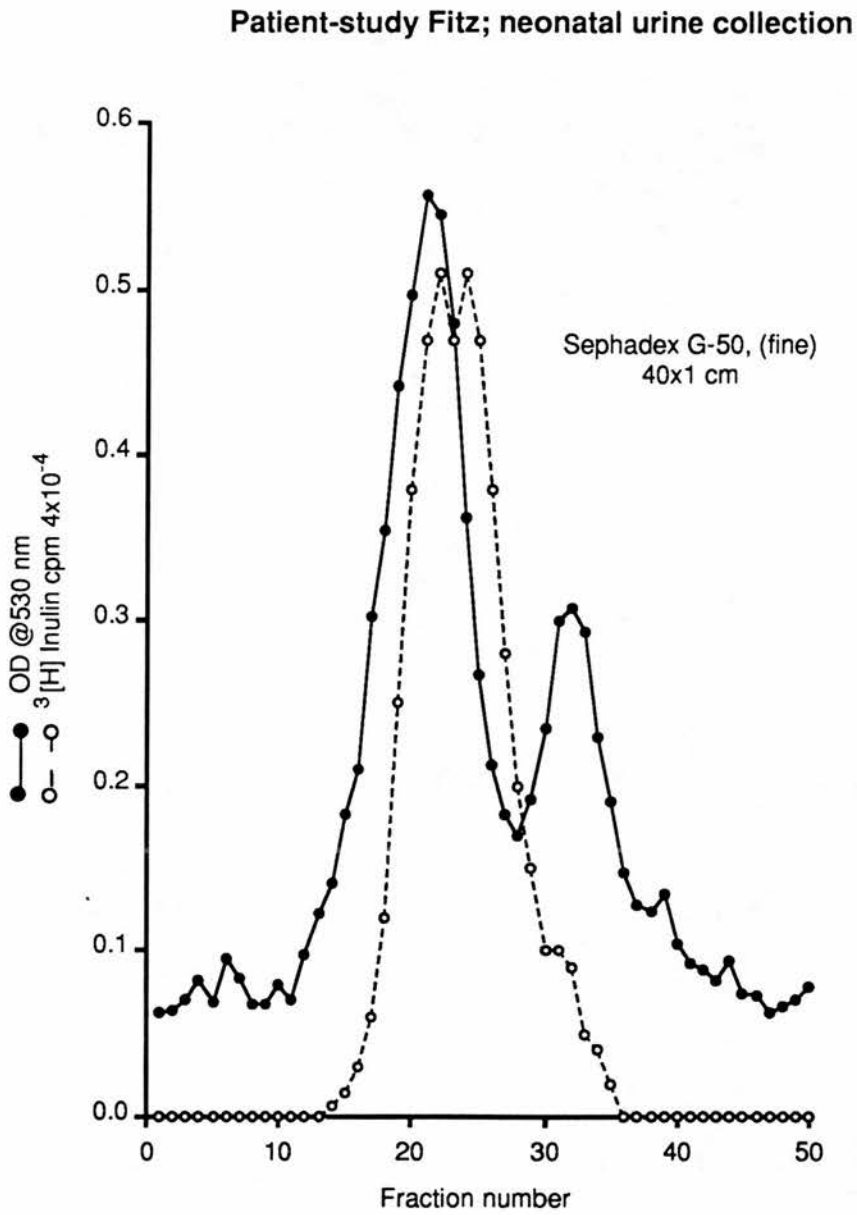


Fig 9. Column chromatography of the representative pooled sample of a total neonatal urine collection (group 1 patient-study)



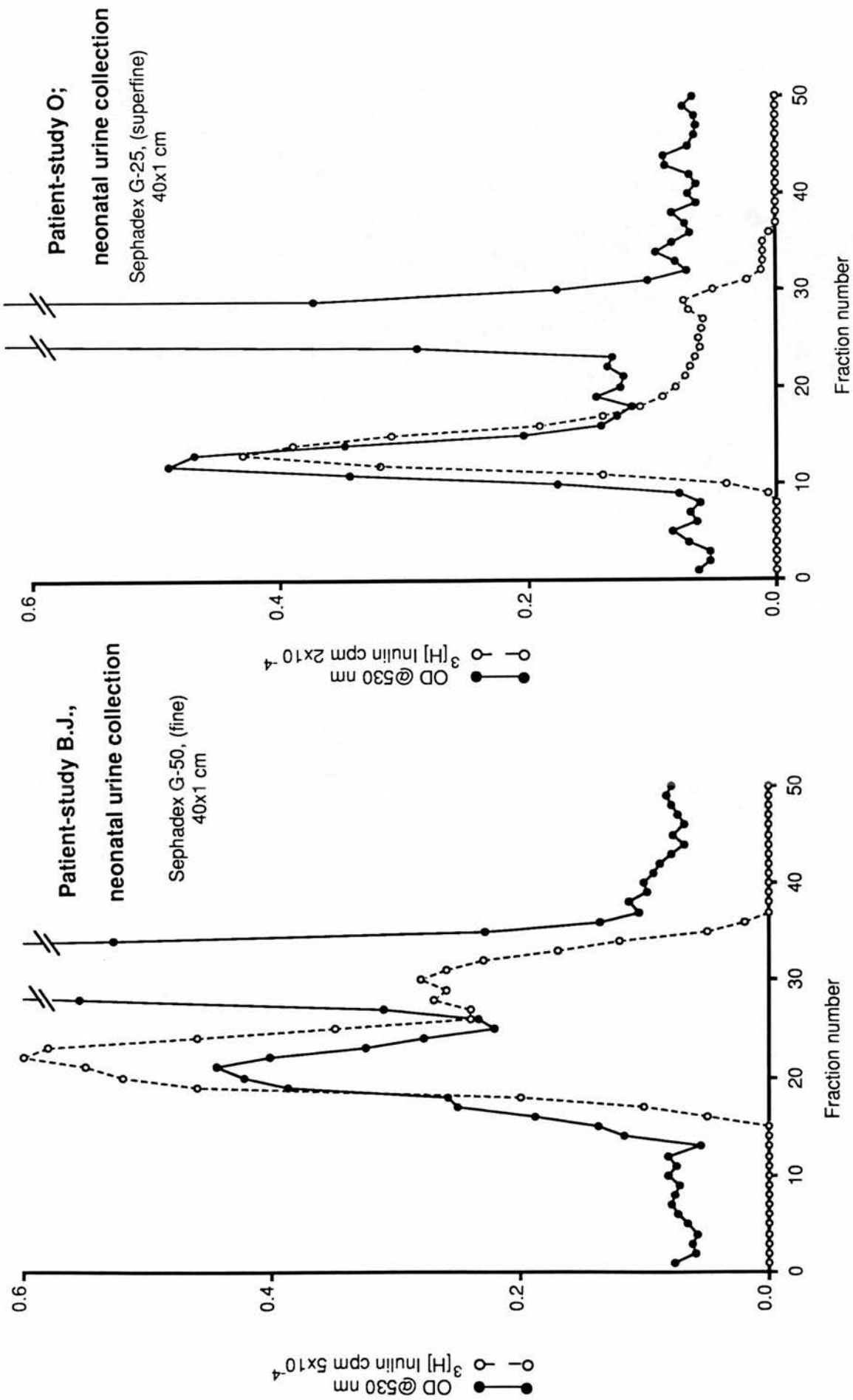


Fig 10. Column chromatography of representative pooled samples of total neonatal urine collections (group 2 patient-studies)

related to the amount of inulin applied to the column in mg.  
by the equation,

$$\begin{array}{lclclcl} \text{Inulin applied} & = & \text{Mean inulin} & \times & \text{Number of} & \times & 0.6 \\ \text{to column } (\mu\text{g}) & & \text{concentration} & & \text{fractions} & & \\ & & (\mu\text{g/ml}) & & & & \end{array}$$

0.6., the given fraction volume

The fraction volume of 600  $\mu\text{l}$  was checked by collecting 6 fractions into weighed vials and then reweighing: mean 594  $\mu\text{g} \pm 1$  ( $\pm$  S.E. of mean).

The accuracy of measuring inulin by peak area measurements on a Sephadex column as above was determined by running the column with several standard amounts of chemical inulin and applying the above procedure to quantify the inulin eluting from the column. This was done for both the Sephadex G-50 and G-25 columns (Fig 11); slope 1.03,  $r = 0.997$ ,  $n = 9$ . The G-25 column could not be used with more than 330  $\mu\text{g}$  of inulin as due to its elution at the void volume, the peak was much narrower and with the larger amounts of inulin, the peak optical density exceeded that of the standards used.

#### Time Course of Inulin Excretion.

Inulin excretion was essentially complete within the time course of the urine collection. This was indicated by two sets of observations. Firstly, in the 6 patient-studies with

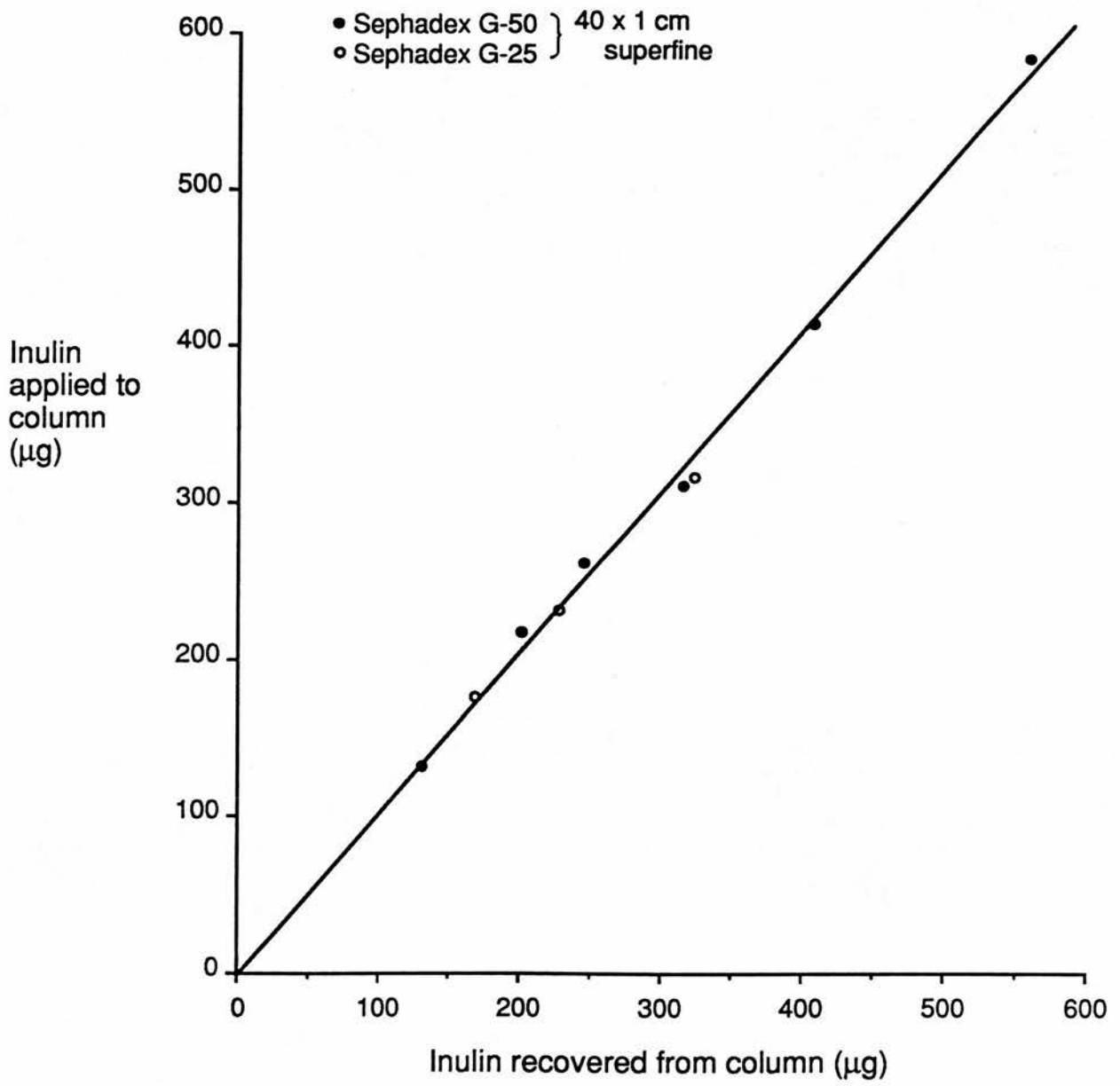


Fig 11. Column recovery of eluted inulin

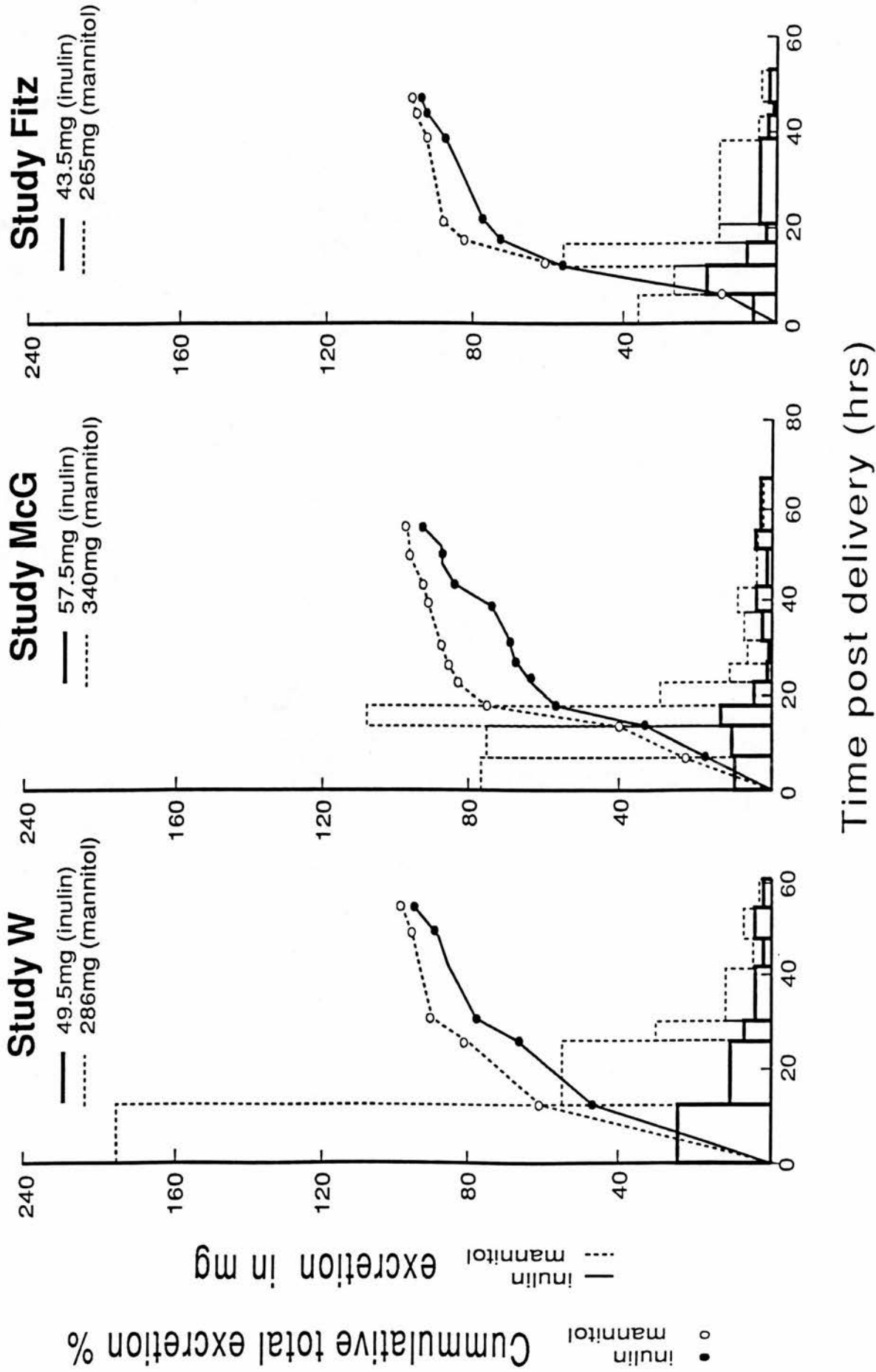


Fig 12. Urinary excretion of mannitol and inulin in 3 of the group 1 patient-studies

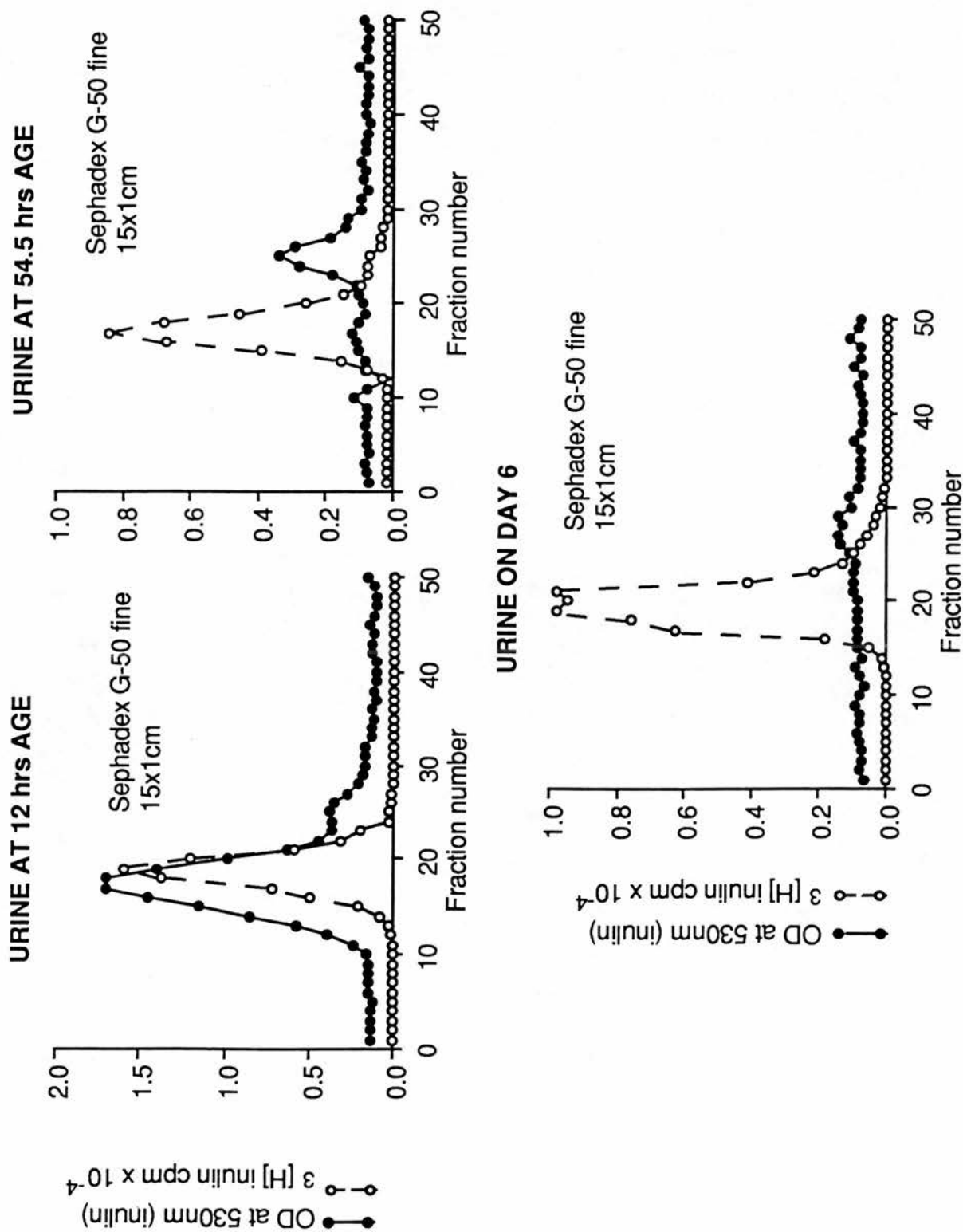


Fig 13. Column chromatography of sequential neonatal urine samples from patient-study W

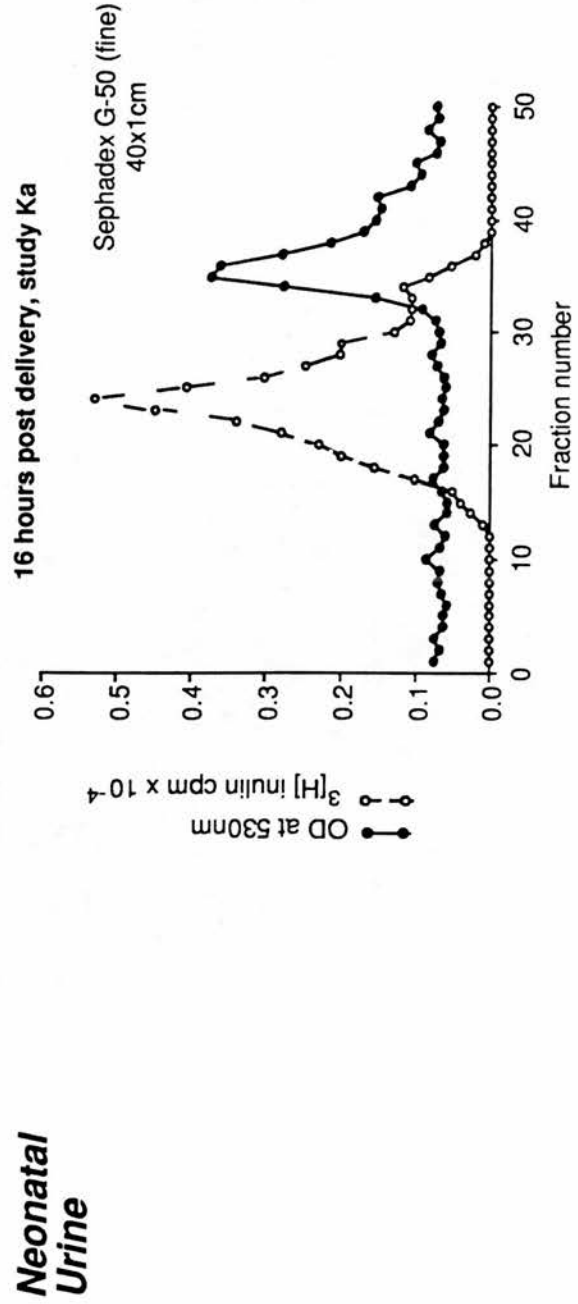
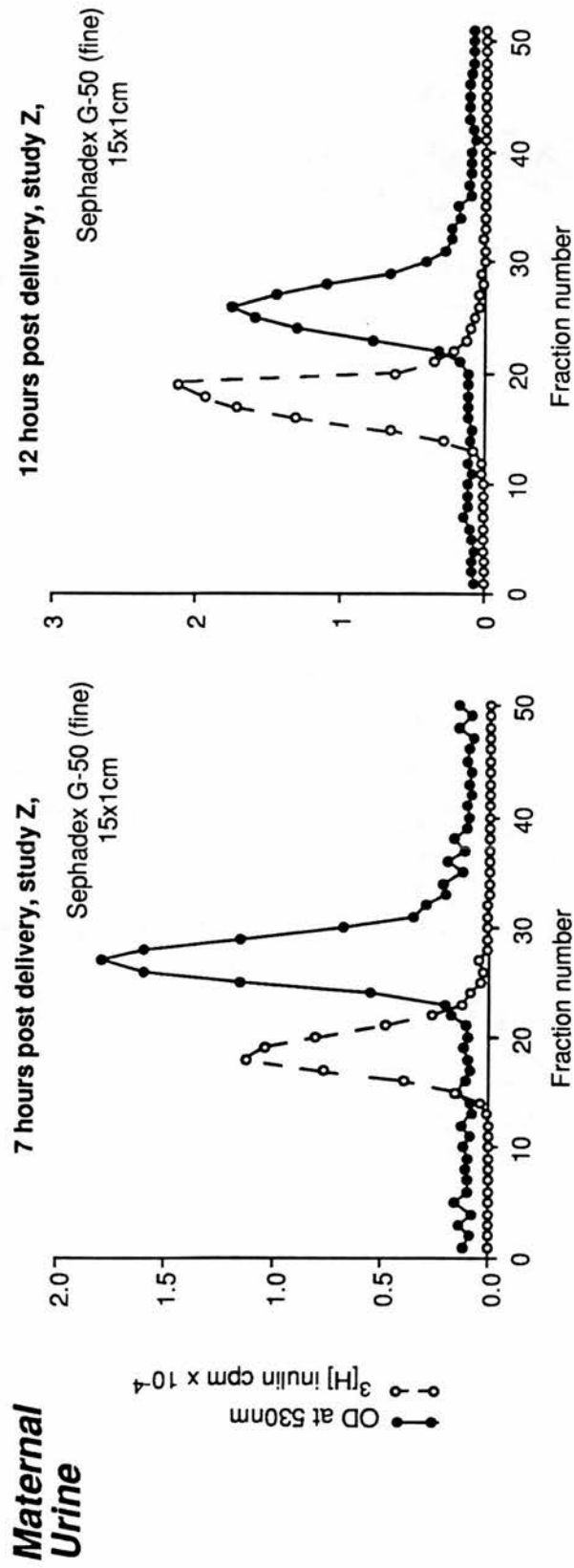


Fig 14. Background cross-reacting material in maternal and neonatal control urine

just inulin and mannitol, its excretion was 90% completed within 48 hours of birth (Fig 12). The concentration profiles all showed the excretion falling to an apparent baseline, suggesting that the excretion had reached background by the time urine collection ceased. Secondly, column chromatography on serial urine samples (Fig 13) could demonstrate no significant inulin in the terminal urine samples corresponding to the baseline, the inulin activity being almost entirely due to the background lower molecular weight material.

#### Nature of Background Material.

In all the samples of maternal and foetal urine and amniotic fluid examined for inulin by the Heyrovsky reaction there was present a lower molecular weight substance that eluted in a fixed position to inulin. In the 15 cm column the interfering substance eluted 7 - 8 fractions after  $^3\text{[H]}$  Inulin, and 10 - 11 fractions after  $^3\text{[H]}$  Inulin in the 40 cm column. This is the same position in which lactulose elutes, indicating a molecular weight of approximately 300 compared with that of 5200 for inulin itself. The column chromatography does not prove that the interfering substance is a single compound; it may be a mixture of different compounds.

This interfering substance or background material was present in both maternal and neonatal control urine (fig 14). The neonatal control urines examined gave blank values of between  $0.2 - 0.5 \text{ mg.ml}^{-1}$  over the first 24 - 36 hours (Fig



7), equivalent to between 20 - 50 mg for a urine output of 100 ml. This is the same order of magnitude as found in the neonatal urine collection of infused patient-studies where mannitol and inulin alone were used, suggesting that inulin degradation made little or no contribution to the interfering substance.

The nature of the background material was investigated by sugar analysis (Dr E Hounsell) and mass spectrometry (Dr A Lawson) at the Clinical Research Centre. Sample fractions of neonatal urine containing the interfering substance were identified and obtained by the standard technique of column chromatography. Sugar analysis showed that the prepared samples contained a 1:1 mixture of galactose and glucose and mass spectrometry though not conclusive, gave a peak with the ion of lactose but with the comment that there were probably other carbohydrates present. There are certainly a whole range of mono-and disaccharides excreted in the urine, some of which are dietary in origin (Pitkanen, 1972, & Bickel, 1961).

#### Stability of the Inulin.

The long term stability of the manufactured inulin was assessed by column chromatography. An aliquot of time expired  $12 \text{ g.dl}^{-1}$  inulin solution that had been manufactured 3 years previously was eluted on Sephadex G-50 using the standard technique above. This was compared with 2 different inulin infusates that had been chromatographed 1 year and 3 months respectively, after manufacture (Fig 15).

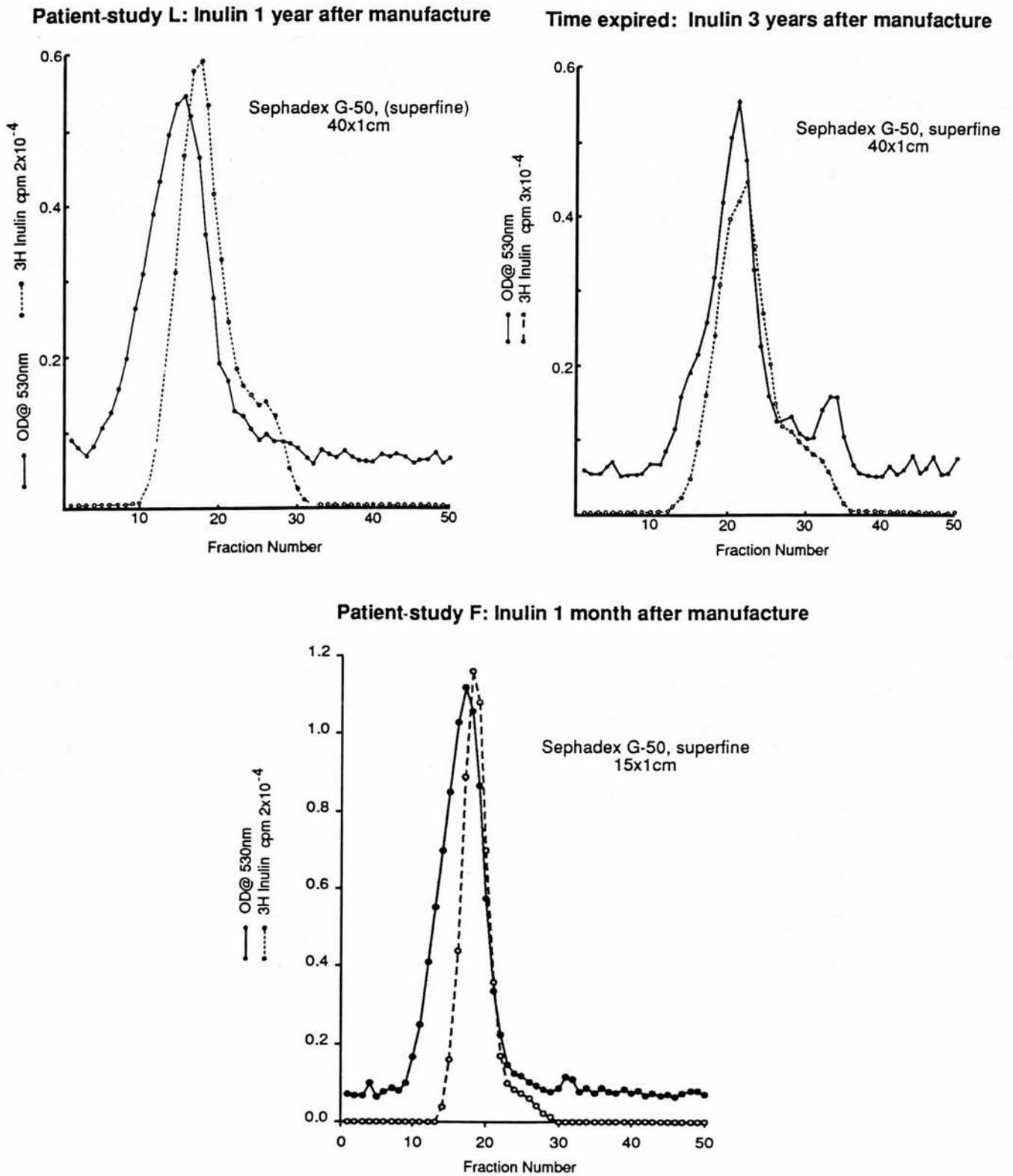


Fig 15. Stability of the infused inulin

This shows that after 3 years approximately 5% of the inulin in solution may undergo decomposition to smaller sized fragments. Inulin chromatographed 1 year and 3 months after manufacture showed no detectable degradation. The manufactured inulin had a shelf-life of one year arbitrarily put on it by the pharmacy department and a new batch of inulin was manufactured after 18 months. The oldest inulin used for patient-studies was therefore 18 months post manufacture, and degradation of the infused inulin therefore minimal.

#### Interaction of inulin with lactulose.

Lactulose gives a positive reaction in the Heyrovsky assay due to its fructose component. Just as with inulin this reaction is linear over the same concentration range (fig 5) and this permits its subtraction when assaying serum and amniotic fluid samples containing both inulin and lactulose to determine the inulin concentration. To confirm that the optical density measurements were additive and there was no interaction between inulin and lactulose, and the subtraction therefore valid, a multiple linear regression analysis was performed on a matrix of a combination of inulin and lactulose concentrations in plasma and water. The analysis was performed by the Department of Medical Statistics, St George's Hospital (Dr M Bland), using the GLIM programme on an IBM computer. The regression analysis was performed with the equation,

$$OD = b_0 + b_1I + b_2L + b_3I.L + E$$

OD : Optical Density

$b_0$  to  $b_3$  : constants

I : inulin, L : lactulose

The analysis showed an interaction achieving significance at the 5% level only, and that ignoring this would cause an error of upto 7% in plasma and 6% for water. This error is the same as the reproducibility of the assays and quite acceptable; there is no obvious reason for the interaction and it is likely that replicate matrices rather than just one matrix would show no significant interaction.

## 2. Lactulose and mannitol.

### Principle.

Lactulose and mannitol were assayed by the method of Laker (Laker, 1979; and Laker & Mount 1980) with modifications. This method used sulphosalicylic acid to deproteinise the plasma, Zerolit DM-F to desalt the plasma supernatant and urine, and air at 50°C to dry the resulting samples. The last traces of water were removed by leaving the samples in an evacuated dessicator containing phosphorous pentoxide for atleast half an hour before derivatisation with pyridine/bis(trimethylsilyl)-acetamide/tri-methylchlorosilane 2/1/1 by volume, the trimethylchlorosilane being necessary to act as catalyst for the silylation. The derivatised samples were analysed by gas

liquid chromatography on a stationary phase of 3% OV-17. The advantages of this method were that it was more robust than the thin layer chromatography used in intestinal permeability studies in adults (Wheeler et al, 1978), the derivatives could be injected directly onto the chromatographic column and be detected by flame-ionization, and lactulose could be measured merely by adjusting the run conditions.

As there was already much expertise and equipment in the department for the measurement of organic acids using capillary gas chromatography, it was decided to use these facilities and modify Laker's method accordingly. The gas chromatographs in the department were 5880A Hewlett Packard gas chromatographs with 10 x 0.001m CP-SIL-5CB fused silica capillary columns.

#### Capillary GC method for mannitol estimation.

Adapting Laker's method to suit capillary gas chromatography involved altering the run conditions and extracting the neutral fraction rather than the acidic fraction (Chalmers & Lawson, 1982).

#### **Assay technique:**

Urine samples (1 ml of the sample itself + 1 ml internal standard,  $\alpha$  methylglucose) were extracted with miniature DEAE sephadex columns (2ml) poured into pipettes and retained by glass wool. Plasma (0.5 ml diluted with 2 ml distilled water) was rendered slightly alkaline by the addition of sufficient 0.1 M

sodium hydroxide to cause a change in litmus paper (to minimise adherence to the cones) and ultrafiltered by centrifugation through Amicon CF ultrafiltration membrane cones before application to the DEAE sephadex column. The neutral fraction was eluted with water (10 ml), shell frozen over a mixture of solid carbon dioxide and ethanol, and then freeze dried. Once this was completed (usually overnight) tetracosane and hexacosane (25  $\mu$ l) were added as GC standards and derivitisation undertaken with trimethylsilylimidazole (200  $\mu$ l for plasma, 500  $\mu$ l for urine). 1  $\mu$ l of the resulting solution was injected onto the column. The carrier gas was helium, flow rate approximately 1.4 ml.min<sup>-1</sup>, injector temperature 200°C, detector temperature 300°C, and oven temperature programmed from 150°C to 250°C at 8°C.min<sup>-1</sup> and held constant for the final 5 minutes. TMSI was chosen because it gave better derivitisation than BSTFA

(bis(trimethylsilyl)trifluoroacetamide) which under certain run conditions gave more than one peak for mannitol and glucose, presumably due to variable anomerisation.

The data analysis was controlled by a BASIC programme 'AUTO CAP RI-X/2+PW'. This saved the raw data and printed a report containing the retention time, retention index, peak height, peak area and the relative peak area. Retention indices were calculated with reference to the cosane standards injected before

each run; the relative peak area was calculated with reference to the tetracosane. The measured concentration of mannitol in each sample was calculated manually with reference to the internal standard  $\alpha$ -methylglucose by the equation,

$$\text{Conc. mannitol} = \frac{\text{peak area}_{\text{ma}} \times \text{conc } \alpha\text{megluc.}}{\text{peak area } \alpha\text{megluc}}$$

ma: mannitol

$\alpha$  megluc:  $\alpha$ -methylglucose

In water, plasma, and urine the standard curve for mannitol was linear over the range of mannitol concentrations 0.01 - 2 mg.ml<sup>-1</sup>. The response factor as calculated from the slope of the standard curve was not unity; each monosaccharide from a range of common monosaccharides examined had a different GC response factor. The reproducibility of the assay was approximately 5%.

Combined gas chromatography-mass spectrometry (Mrs BM Tracey) was used to confirm that the hexatrimethylsilyl derivative of mannitol was being formed (Laker & Mount, 1980), that the same derivative was being formed when both urine and plasma were derivatized, and that it was mannitol that was being measured in these fluids.



Gas chromatographic method for the measurement of mannitol and lactulose.

As the study gained momentum, it became apparent that there was not the time available on the capillary gas chromatograph to analyse the samples for both mannitol and lactulose. The assays were therefore transferred to a Pye Series 204 gas chromatograph using a 3 x 0.005 m column of 3% OV17 and were undertaken in conjunction with Miss D. Copas and Dr MJ Landon.

**Reagents:**

The saccharides were obtained from the Sigma Chemical Company, Poole, Dorset, the trimethylsilylimidazole from Pierce Chemicals, Chester, Cheshire, DEAE Sephadex A-25 from Pharmacia Ltd, Milton Keynes, Buckinghamshire, and ultrafiltration cones from the Amicon Corporation, MA, U.S.A.

Stock solutions of mannitol and lactulose 5 mg. ml<sup>-1</sup>, and  $\alpha$ -methylglucose and D+ trehalose 10mg.ml<sup>-1</sup> were prepared and used to make up the standards 0, 0.25, 0.5, 1, and 1.5 mg.ml<sup>-1</sup>. in either water if urine was being assayed or pooled cord sera if serum was being assayed.

**Assay technique:**

(1) Urine. To 0.5 ml of a 1:5 dilution of the sample was added 0.1 ml of  $\alpha$ -methylglucose (5mg.ml<sup>-1</sup>)

and 0.1 ml of D+ trehalose ( $5 \text{ mg.ml}^{-1}$ ) and 0.3 ml of distilled water. The  $\alpha$  methylglucose was the internal standard for the mannitol assay and the D+ trehalose the internal standard for the lactulose assay. Samples were prepared in duplicate. The samples were extracted by adding 2 ml of wet DEAE Sephadex, mixing, and centrifuging for 5 minutes. The supernatant was removed and 200  $\mu\text{l}$  blown down at  $50^{\circ}\text{C}$  under nitrogen for at least 30 minutes. Derivatisation was effected by the addition of 50  $\mu\text{l}$  of TMSI at  $60^{\circ}\text{C}$  for at least 30 minutes. 1  $\mu\text{l}$  was injected on to the column. Mannitol estimations were run with temperatures of column  $180^{\circ}\text{C}$ , injector  $250^{\circ}\text{C}$ , and detector  $300^{\circ}\text{C}$ . Lactulose estimations were run with a column temperature of  $250^{\circ}\text{C}$ , injector temperature of  $300^{\circ}\text{C}$  and detector  $350^{\circ}\text{C}$ .

(2) Serum and amniotic fluid. To 250  $\mu\text{l}$  of serum, 50  $\mu\text{l}$  of  $\alpha$  methylglucose ( $10 \text{ mg.ml}^{-1}$ ), 50  $\mu\text{l}$  of D+ trehalose and 150  $\mu\text{l}$  of distilled water were added and mixed. The samples were deproteinised by ultracentrifugation using Amicon MP 51 ultrafiltration cones, and then analysed in the same way as for urine.

The lower limits of the assays were  $0.015 \text{ mg.ml}^{-1}$  for mannitol and  $0.04 \text{ mg.ml}^{-1}$  for lactulose; reproducibility was 5%.

### 3. CrEDTA.

CrEDTA was assayed by electrothermal atomisation atomic absorption spectrophotometry using totally pyrolytic graphite cuvettes on a Pye Unicam SP9-800 atomic absorption spectrometer with Pye Unicam video furnace and autosampler by Dr A Taylor at the University of Guilford (Taylor & Green, 1988). The urine was transferred immediately after collection into chromium free tubes and stored frozen until assayed. Serum was obtained by allowing the blood to clot and then removing the serum to be stored frozen until assayed; all the tubes and pipettes used were chromium free.

### Estimation of molecular radius of CrEDTA.

The molecular radii of mannitol, lactulose and inulin are well established in the literature (Normand, Olver, Reynolds, et al, 1971) but this is not the case for CrEDTA. CrEDTA has been used in intestinal permeability measurements (Maxton et al, 1986), but in this and similar studies, the molecular mass of CrEDTA (387) has been taken as a measure of molecular size; any disparity between molecular weight and molecular size for CrEDTA was unimportant as long as the molecular radius was the same as, or greater than that of lactulose, as both had similar permeability profiles.

The effective molecular radius of CrEDTA for diffusion through an epithelial membrane will be influenced by the hydration shell of the complex and whether there is discrimination by the membrane on the basis of charge as CrEDTA is a weakly charged anion. These considerations do

not apply to the other three permeants used in the study. Gel filtration chromatography is a well established technique for determining molecular size using standards of known molecular size to construct a calibration curve against which the molecular size to be determined can be calculated. Boyd et al, (1976) determined the  $K_{av}$  of erythritol, mannitol, and sucrose, all of whose molecular radii are well established, and that of CrEDTA on Sephadex G-15 chromatography (30 x 1 cm column). The  $K_{av}$  for CrEDTA was smaller than that for sucrose indicating a larger molecule size than the largest saccharide, sucrose. Linear extrapolation from the three saccharides was therefore required to estimate an effective molecular size for CrEDTA. To properly measure the molecular size of CrEDTA requires that it be within the range of the molecular size range of the markers being used. The chromatography was therefore repeated but with a larger molecular size marker at the upper limit. Stachyose, mol. wt. 666, a tetrasaccharide ( $\alpha$ -D-Gal- $\alpha$ -D-Gal- $\alpha$ -D-Glu- $\beta$ -D-Fru), was chosen as the upper molecular sized marker because of its larger size and its terminal fructose residue allowing detection by the Heyrovsky reaction.

Sephadex was prepared in a column 15 x 1 cm. De-gassed phosphate buffered saline, pH 7 (NaCl 9.3mM, KCl 0.2 mM,  $\text{Na}_2\text{HPO}_4$  0.05mM, and  $\text{KH}_2\text{PO}_4$ ) was used to both equilibrate and elute the column. A solution containing  $^3\text{[H]}$  inulin  $2.2 \mu\text{Ci.ml}^{-1}$ ,  $^{51}\text{[Cr]}$  EDTA  $1.6 \mu\text{Ci.ml}^{-1}$ ,  $^{14}\text{[C]}$  mannitol  $21 \mu\text{Ci.ml}^{-1}$ , and  $^3\text{[H]}$  water  $1.7 \mu\text{Ci.ml}^{-1}$  (all Amersham

International PLC, Amersham, England), stachyose  $4.2\text{mg.ml}^{-1}$  (Aldrich Chemical Company, Dorset, England) and lactulose  $4.2\text{ mg.ml}^{-1}$  (Sigma Chemical Company, Dorset, England) was prepared.  $70\text{ }\mu\text{l}$  of this solution was applied to the column and eluted at a flow rate of  $2\text{ ml.min}^{-1}$ .  $50 \times 600\text{ }\mu\text{l}$  fractions were collected. An aliquot of each fraction was assayed by the method of Heyrovsky (1956) and a further aliquot mixed with  $4\text{ ml}$  of NE265 liquid scintillant and counted sequentially on a LKB 1215 Rackbeta and LKB 1280.

Both lactulose and stachyose were identified by a positive Heyrovsky reaction. The fractions with the highest activity of  $^3\text{[H]}$  water and  $^3\text{[H]}$  inulin were taken as  $V_t$ , the total liquid volume, and  $V_o$ , the void volume, respectively.  $V_e$ , the elution volume, was taken as the fraction with the highest activity of the respective solute.

#### Manufacturing protocols.

Inulin, lactulose, and CrEDTA were prepared for parenteral use and tested accordingly by the Pharmacy Department at Northwick Park Hospital, Harrow.

#### 1. Inulin.

Inulin was obtained as the pure powder from Koch-Light Ltd, (Haverhill, Suffolk). A sterile solution of  $12\text{g.dl}^{-1}$  in  $0.8\text{g.dl}^{-1}$  sodium chloride solution was made according to the British Pharmacopoeia (British Pharmacopoeia 1980). Using aseptic techniques  $540\text{g}$  of inulin powder and  $36\text{g}$  of sodium chloride was dissolved at  $75^\circ\text{C}$  in  $4.5\text{L}$  of sterilised

distilled water. This solution was sterilized by aseptic filtration through 0.22  $\mu$  filters before being dispensed into individual sealed bottles each containing 150ml. Pyrogen testing was performed in rabbits before the resulting inulin solution was passed fit for human intravenous use. With storage the inulin precipitated into large flakes that were re-dissolved by heating to 80°C for half an hour until no particulate matter remained. The solution was then allowed to cool to room temperature before being used for a patient-study later that day.

## 2. Lactulose.

Lactulose was prepared from the pure crystalline powder which was a gift from Duphar Laboratories Ltd, Southampton. 250 g was dissolved in 1667 ml of sterile water for injection. The resulting solution was sterilised by filtration through 0.22  $\mu$  filters and checked for sterility and toxicity. The final solution contained lactulose 15 g. dl<sup>-1</sup>.

## 3. Mannitol

Mannitol was obtained as a 20 g.dl<sup>-1</sup> solution ready for parenteral use (Polyfusor, Boots Co PLC, Nottingham).

## 4. CrEDTA.

CrEDTA suitable for parenteral use was prepared by the same method used by Amersham International PLC to prepare <sup>51</sup>CrEDTA (manufacture sheet kindly supplied by Amersham

International PLC) with the difference that the starting material was chromium (III) and with the obvious omission of radiolabelled chromium. The chromium EDTA complex was formed by heating an excess of the disodium salt of ethylenediaminetetraacetic acid (EDTA) with the chromium salt and the subsequent addition of base to ensure complexation is complete.

50 mg chromium (III) in the form of chromium chloride hexahydrate was dissolved with disodium edetate in 350 ml of fresh distilled, sterile water. The resulting solution was heated for one hour in a steam bath and within 10 minutes the solution turned a pale deep purple colour. The solution was then cooled and the pH adjusted to 3.5 - 4.0 with 1M sodium hydroxide and the final volume brought to 500 ml before being dispensed into 5 ml ampoules. The ampoules were then sterilised by autoclaving and tested for sterility.

#### Preparation of the final infusate.

For studies using just mannitol and inulin, 150 ml of already prepared inulin ( $12\text{g.dl}^{-1}$ ) was dissolved by heating in a water bath at approximately  $80^{\circ}\text{C}$  until the inulin was fully dissolved and the solution crystal clear. To this was aseptically added 150 ml of mannitol ( $20\text{ g.dl}^{-1}$ ) to give the administered infusate of inulin  $6\text{ g.dl}^{-1}$  and mannitol  $10\text{ g.dl}^{-1}$ .

For the studies using the four permeants, mannitol, lactulose, CrEDTA, and inulin it was felt more appropriate that the pharmacy department should prepare the final



solution under proper sterile conditions as three additions were required rather than one as with mannitol and inulin alone. The infusate was prepared by the addition of 150 ml of inulin,  $12\text{g.dl}^{-1}$ ; 10 ml of CrEDTA,  $400\text{ }\mu\text{mol.l}^{-1}$ ; 200 ml lactulose,  $15\text{ g.dl}^{-1}$ ; and mannitol 150 ml,  $20\text{ g.dl}^{-1}$ . The final infusate with the four permeants thus consisted of mannitol  $6\text{ g.dl}^{-1}$ , lactulose  $6\text{ g.dl}^{-1}$ , CrEDTA  $8\text{ nmole.ml}^{-1}$ , and inulin  $3.6\text{ g.dl}^{-1}$ . The different infusion regimes detailed on p 45 were to allow similar amounts of inulin and mannitol to be given to the two groups of patient-studies.

## Chapter 4

## Mathematical theory and analysis.

Derivation of permeability constants.

Transfer across the placenta like all biological membranes is a two way process with transfer or flux,  $J$ , occurring in both directions across the placental membrane, namely  $J_{m \rightarrow f}$  and  $J_{f \rightarrow m}$ . Net transfer to the foetus, which underlies its ability to accumulate nutrients is an absolute prerequisite for growth, requires that flux from mother to foetus exceeds the back flux from foetus to mother, namely

$$J_{net} = J_{m \rightarrow f} - J_{f \rightarrow m}$$

In the case of a foetal excretory metabolite, for example urea, the equation expressed in this way would result in a negative value for  $J_{net}$ .

Transfer by free diffusion across the placental membrane can be described by the Fick equation which relates the uni-directional flux per unit area of membrane to the concentration on the donor side and a constant,  $P$ , the permeability for that membrane with units of  $\text{length} \cdot \text{time}^{-1}$ . Thus,

$$J_{m \rightarrow f} = C_m \cdot P$$

$$J_{f \rightarrow m} = C_f \cdot P$$

and  $J_{\text{net}} = (C_m - C_f) \cdot P$

where  $C_m$  : maternal concentration

$C_f$  : foetal concentration

In whole organ studies the area of transfer cannot easily be measured and the equation can be multiplied on both sides by a surface area per unit weight term to produce a more physiologically useful equation,

$$J_{\text{net}} \cdot S = (C_m - C_f) \cdot P \cdot S$$

$J_{\text{net}}$  : net flux per  $\text{cm}^2$  membrane

$C_m$  : maternal concentration,  $\text{mol.ml}^{-1}$

$C_f$  : foetal concentration,  $\text{mol.ml}^{-1}$

$P$  : permeability  $\text{cm.sec}^{-1}$

$S$  : surface area per gram  $\text{cm}^2.\text{g}^{-1}$

The above equation can be rewritten in either of two forms which relate net transfer to the whole placenta or per gram placenta by a constant, the placental clearance  $K$  or  $K_{\text{Wt}}$  respectively,

$$P \cdot S \cdot \text{Wt} = \frac{J_{\text{net}} \cdot S \cdot \text{Wt}}{C_m - C_f} = K (\text{ml.min}^{-1})$$

$$P \cdot S = \frac{J_{\text{net}} \cdot S}{C_m - C_f} = K_{\text{Wt}} \text{ (ml.min}^{-1}\text{.g}^{-1}\text{)}$$

The measured placental clearance,  $K$ , is influenced not only by the placental permeability, but also by the placental blood flow. The importance of studying permeants which have low placental clearances in relation to placental blood flow (i.e. transfer is membrane limited) is thus clear if the data is to be analysed in terms of membrane structure (see section on pore theory).

To measure placental clearance or permeability, net transfer and the concentration gradient down which this transfer occurred must be measured. Fick's law applied to transfer across the placenta requires the concentration gradient to be measured to be that across the placental membrane. Because the transfer of the four permeants being studied is so low in comparison with the blood flows on either side of the placenta, the maternal and foetal concentrations on either side of the placental membrane will approximate to the mixed venous concentrations that can be measured peripherally. The mean transplacental concentration gradient will thus equal the difference between the maternal and foetal blood profiles (fig 16) determined from maternal venous blood, and cord blood taken at the time of delivery. The foetal profile is constructed by joining the level in cord blood which is at the end of the infusion with the level at the start of the infusion which is obviously

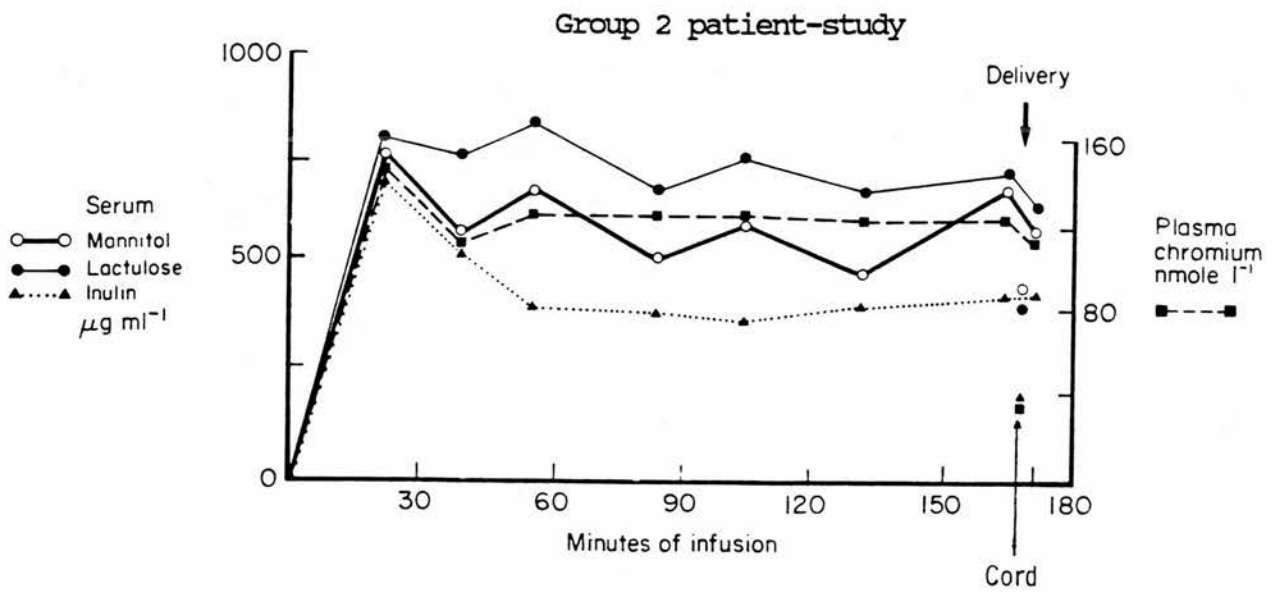
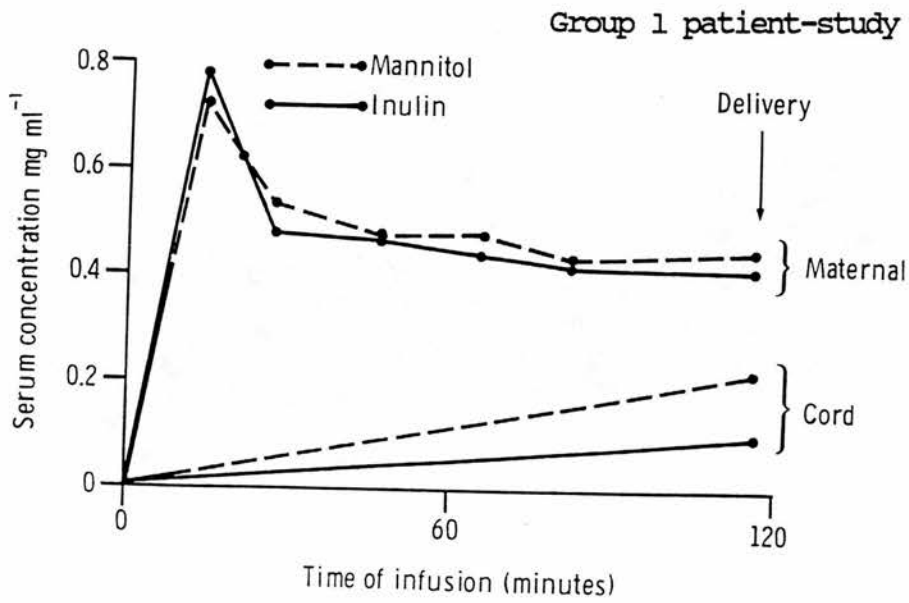


Fig 16. Maternal and cord serum profiles from a group 1 and group 2 patient-study

zero for the exogenous permeants.

For substances that are inert, non-metabolisable, and wholly and solely cleared by renal excretion (the four permeants in the study), net flux to the foetus during maternal infusion will equal their subsequent neonatal urinary excretion.

The equation used to calculate placental clearance is therefore,

$$\text{Clearance, } K, \text{ ml.min}^{-1} = \frac{\text{Net flux, mg}}{\text{Mean conc diffce. x Duration}} \\ \text{mg.ml}^{-1} \qquad \qquad \text{infusion} \\ \qquad \qquad \qquad \text{min}$$

#### Passive permeability and the pore theory.

The Fick equation gives the rate of diffusion down a concentration gradient,

$$\frac{dv}{dt} = J_s = \frac{-D_s \cdot A \cdot dC}{dx} \dots\dots\dots(1)$$

where  $dv/dt$  is the rate of diffusion in  $\text{mol.sec}^{-1}$ ,  $J_s$ ,  $dC/dx$  the concentration gradient down which diffusion is occurring in  $\text{mol.ml}^{-1}.\text{cm}^{-1}$  (the negative sign indicates that diffusion is taking place in the direction of the decreasing concentration), and  $A$  the area of the plane of solution at right angles to the movement. The constant,  $D_s$ , is the

diffusion coefficient which in the case of a solute free in solution is the coefficient of free diffusion in water with units of  $\text{cm}^2.\text{sec}^{-1}$  or  $\text{cm}.\text{sec}^{-1}$ .

The Fick equation can equally be applied to the passage of substances across membranes by passive diffusion. The constant,  $D_m$ , is however the diffusion coefficient in that membrane,  $dC_m/dx$  the gradient just within the membrane at its outer and inner borders, and there is an additional term  $PT$ , the partition coefficient, the ratio of the concentration of the solute in the membrane to that in aqueous solution when these are at equilibrium,

$$J_m = \frac{D_m \cdot A_m \cdot dC_m}{dx_m} \cdot PT \quad \dots\dots\dots(2)$$

where  $J_m$ : rate of diffusion

For any given cell membrane, thickness will be the same for all solutes so that permeability will be determined by the two constants for that membrane, the partition coefficient and diffusion coefficient. For lipid soluble substances, the direct proportionality between permeability and the partition coefficient suggests that such substances cross the membrane by dissolving in the lipid component of the membrane (Fig 3). For small water soluble molecules this relationship does not hold, and infact diffusion is related primarily to molecular size, the permeability coefficient being inversely proportional to the cube root of the



molecular volume. Thus there arises the supposition that hydrophilic molecules cross cell membranes largely or solely by passive diffusion through water-filled pores or channels, the so-called pore theory.

The thermodynamic analysis of diffusion through pores in membranes, both biological and synthetic was unified by Solomon in his now classic paper (Solomon, 1968) in which Fick's original equation for diffusion was modified to describe diffusion across membranes that still took place in solution but limited to aqueous pores. The concentration difference was measured in the opposite direction to produce a change in sign, and a term  $A_s$  was introduced to describe the additional restriction to free diffusion in solution introduced by the pores,

$$J_s = D_s \cdot A_s \cdot \frac{dC}{dx} \quad \dots\dots\dots(3)$$

The term  $A_s$  contained two components. The first,  $A_p/A$  the ratio of total pore area to the total membrane area, and a second term  $A_{sd}/A_p$ , which takes account of the frictional resistance between the solute and the walls of the pore (the Faxen term) and the steric hindrance at the pore entrance (the Ferry term). Solomon (1968) agreed with previous authors that the equation derived by Faxen is to be preferred to that used by Pappenheimer in describing the friction between solute molecules and the walls of the pore

to give,

$$\frac{A_{sd}}{A_p} = (1 - \alpha)^2 \cdot (1 - 2.104\alpha + 2.09\alpha^3 - 0.95\alpha^5) \dots\dots\dots(4)$$

where  $\alpha = a/r$  the ratio of the radius of the solute molecule  $a$ , to the pore radius,  $r$ . Applying the correction factor,  $A_s$ , to the relationship between the permeability coefficient and the diffusion coefficient (equation 3) allows calculation of the pore radius from the permeability of the membrane,

$$K_s = D_s \cdot \frac{A_m}{dx} \cdot \frac{A_p}{A_m} \cdot \frac{A_{sd}}{A_p} \dots\dots\dots(5)$$

$$= D_s \cdot \frac{A_p}{dx} \cdot \frac{A_{sd}}{A_p}$$

$$= D_s \cdot \frac{A_p}{dx} \cdot (1 - \alpha)^2 \cdot (1 - 2.104\alpha + 2.09\alpha^3 - 0.95\alpha^5)$$

$$\dots\dots\dots(6)$$

Pore theory describes the behaviour of small hydrophilic molecules in terms of 'notional ' pores without implying any anatomical or structural characteristics.

#### Estimation of molecular size.

This was performed on Sephadex gel filtration

chromatography. Separation by gel filtration chromatography, of which Sephadex is one type, depends on the ability of the sample molecule to enter pores contained within the gel. These entrap some of the eluent, the stationary phase, and access to this phase is governed by molecular size. The smaller the molecule the more readily it enters the gel pores, and thus the slower it moves through the column as it spends longer in the pores. As Sephadex is a bead-formed gel prepared by cross-linking dextran, the differing degree of cross-linking allows beads with different pore sizes and thus different fractionation ranges to be constructed (Pharmacia, 1985).

$K_{av}$  is one of the measures of the volume of the stationary phase available for a given solute. It is calculated from the relationship,

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where  $V_o$  is the void volume, the volume of the mobile phase determined by the elution volume of a substance that is excluded by the gel,  $V_e$  the elution volume, the volume at which the sample elutes, and  $V_t$  the total liquid volume or volume of the packed bed, determined by the elution volume of a substance that freely enters the gel. Because access to the stationary phase is governed by molecular size  $K_{av}$  is also a measure of the molecular size of the given solute species.

In attempting to derive a mathematical relationship between  $K_{av}$  and molecular radius (R) various models for the gel have been tried. Laurent and Killander (1964) assumed the gel network to be composed of rigid rods randomly arranged with the pores formed by this meshwork. A good correlation was found between  $K_{av}$  and molecular radius with this model but the relationship was sigmoidal. Siegel and Monty took the same random meshwork model but linearized the equations by logarithmic transformation to derive the relationship (Rodbard, 1976),

$$\sqrt{-\log(K_{av})} = a + b.r$$

where a & b are constants  
and r = molecular radius

#### Compartmental analysis.

Compartmental analysis was used to quantify the error introduced into the calculation of clearance by the assumption of a linear rise in foetal permeant concentrations whereas the rise would be more properly described by an exponential function.

For this analysis the extracellular fluid space of mother and foetus are considered as two compartments separated by a permeable membrane. If a constant concentration of solute is maintained in one compartment, the maternal compartment  $C_m$ ,

transfer to the other compartment, the foetal compartment  $C_f$ , will be given by the exponential function (Moll, 1981),

$$C_f = C_m(1 - e^{-kt}) \quad \dots\dots\dots(7)$$

where  $t$  is time

The maternal-foetal profile can be expressed in one of two ways: in units of absolute concentration against time, or as the ratio of foetal:maternal concentration plotted against time. In the former case the maternal:foetal concentration gradient is the area between the two curves, while in the latter case the maternal-foetal gradient is the area between the curve of the ratio of foetal:maternal concentration and unity. Expressing the foetal concentration as a ratio of the maternal concentration allows the application of compartmental analysis to express the difference between an exponential and a linear rise in foetal permeant concentrations as a function of foetal:maternal permeant concentration at the time of delivery.

The maximum ratio of  $C_f:C_m$  found experimentally at the time of delivery was 0.7 for mannitol (the smallest permeant studied and the most permeable). Applying equation (7) to this ratio gives a corresponding value of  $kt$  equals 1.2. The difference between a linear rise in the ratio of  $C_f:C_m$  and an exponential rise described by the above exponential is equal to the area between the respective curves. This can be

measured by integration of these curves between the limits for  $kt$  of 0 and 1.2,

$$\frac{C_f \cdot dt}{C_m} = \int_0^{1.2} (1 - e^{-kt}) \quad \dots \text{for exponential curve}$$

$$= 0.5$$

$$\frac{C_f \cdot dt}{C_m} = 0.5 \times 1.2 \times 0.7 \quad \dots \text{for linear curve}$$

$$= 0.42$$

The error caused by assuming a linear as opposed to an exponential increase is then given by comparison of the areas between the above curves and the boundaries of unity equilibrium on the  $C_f:C_m$  axis and 1.2 on the  $kt$  axis. This is found by subtracting the area below the respective curves (0.5 for the exponential curve, 0.42 for the linear curve as determined above) from  $1 \times 1.2$ , which gives an area of 0.7 for the exponential increase and 0.78 for the linear increase. The error is thus,

$$\frac{0.78 - 0.7}{0.7} = 11\%$$

A linear rise therefore underestimates the ratio  $C_f:C_m$

for the duration of the maternal infusion and this corresponds to an overestimate of 11% in the mean maternal-foetal concentration gradient for a ratio of 0.7 at delivery.

#### Statistical analyses.

Statistical evaluation of the results was carried out by means of a statistical package on an Apple II Europlus computer or a Zenith personal computer. These allowed the mean, standard deviation, and standard error, to be determined for a set of results, a Student's t test to be performed on two sets of data, and a paired t test or Wilcoxon paired rank sum test to be performed on paired data. The programmes also allowed the calculation of a correlation coefficient between two sets of data using the method of least squares analysis. The correlation coefficient was converted into a two-tailed level of significance by reference to Snedecor and Cochran (1980).

## Chapter 5.

## Results.

## 1. Patient details.

Thirteen patient-studies were completed, 6 with infusions of mannitol and inulin (group 1) and 7 with mannitol, lactulose, CrEDTA, and inulin (group 2). The reasons for delivery by elective caesarean section are given in Table 4.

Table 4. Reasons for delivery by elective caesarean section.

Patient	Reason for caesarean section
W	Unstable lie at term with maternal age 39 years
McG	Breech presentation with borderline pelvimetry in primigravida
Fitz	Breech presentation in primigravida with 3 years of primary infertility
L	Breech presentation in primigravida aged 39 years
l	Oblique lie, large baby, and failure of the foetus to descend
K	Two previous LSCS
BJ	Three previous LSCS
Nas	Two previous LSCS
Flet	Two previous LSCS
O'R	Two previous LSCS
Sha	Two previous LSCS for cephalopelvic disproportion
Oreg	Two previous LSCS, the first an emergency for foetal distress
Mag	Breech presentation with borderline pelvimetry in a primigravida

Maternal clinical details are listed in Table 5. Maternal age ranged from 20 - 39 years (mean 32). Ten sections were



Table 5. Maternal clinical details in the 13 patient-studies

Patient-study	W	McG	Fitz	L	Il	K	BJ	Nas	Flet	O'R	Sha	Oreg	Mag
Maternal details:													
Age (yrs)	39	20	30	39	33	31	39	33	36	20	29	34	36
Ht (cm)	164	164	170	164	162	166	153	161	161	165	164	151	163
Wt (kg)	78	67	87.5	77.5	78	58	63	66	66.5	72.6	65	77	56.4
Parity	3	0	0	0	2	2	3	2	2	0	2	2	0
Anaesthetic	GA	GA	GA	Epi	GA	GA	GA	GA	GA	Epi	Epi	GA	GA
Smoking	0	+	±	0	0	+	±	+	+	0	0	0	0
Gestation (completed weeks)	40	38	39	40	38	37	38	38	38	38	38	38	40

GA., General anaesthetic  
 Epi., Epidural  
 Smoking; 0., non-smoker  
 ±., smoker  
 ±., stopped during pregnancy

carried out under general anaesthetic and 3 under epidural. One delivery (K) was at 37 completed weeks of gestation, the rest were between 38 and 40 weeks.

All infants were delivered in good condition with APGAR scores of 9 or 10 at both 1 and 5 minutes. None required any resuscitation. No problems were detected at the first day and discharge post-natal checks and mothers and babies were discharged at the scheduled time. Neonatal clinical data are contained in Tables 7 and 9. Five of the study infants were exclusively breast fed, 5 exclusively bottle, and three received a mixture of breast and bottle feeds. Six of the infants were male and 7 female.

## 2. Blank values.

(i) Inulin. Blank values for inulin in cord blood ranged from 20-30  $\mu\text{g}.\text{ml}^{-1}$ , mean 25 ( $n=6$ ) and in amniotic fluid from 10-20  $\mu\text{g}.\text{ml}^{-1}$ , mean 15 ( $n=3$ ). The pre-infusion maternal blood sample was used to provide the maternal serum blank value which was subtracted from the subsequent maternal serum values; mean value of the maternal serum blank was 35  $\mu\text{g}.\text{ml}^{-1}$  (range 0 - 60).

The cross-reacting substance(s) in neonatal urine were separated from the true inulin by column chromatography. The neonatal excretion rate of inulin fell to a 'baseline' towards the end of the urine collection suggesting residual interfering material (Fig 7). This was confirmed by column chromatography on the terminal neonatal urine specimens from 3 of the group 1 patient-studies (Fig 17) which showed

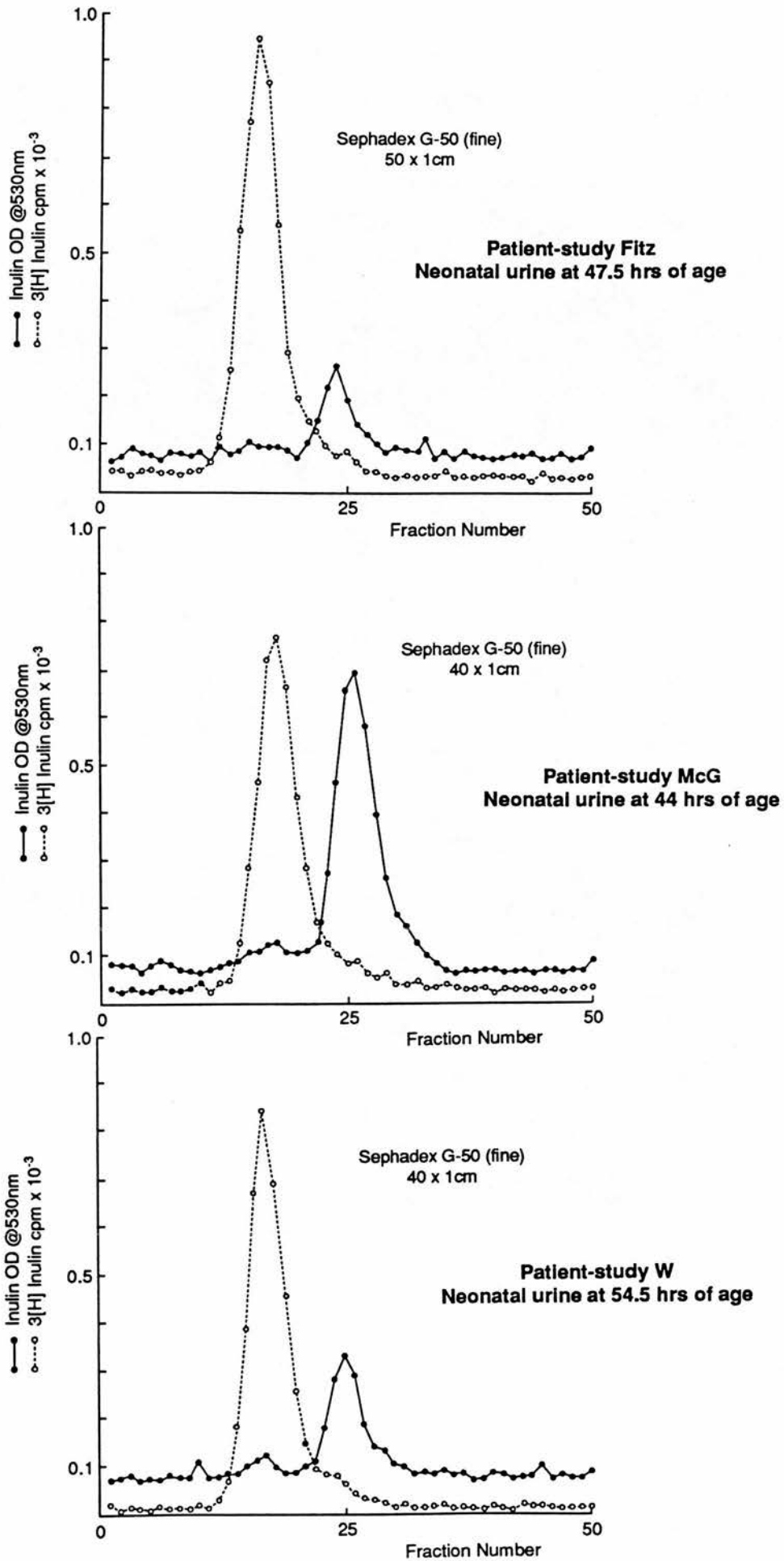


Fig 17. Chromatography of the terminal urine sample of the neonatal urine collection in 3 patient-studies

minimal inulin activity present. No inulin was identified in neonatal urine samples collected at six days of age, approximately 3 days after the period of urine collection was finished, though the interfering substance appeared to be less than in the terminal urine sample from the period of urine collection. Column chromatography also allowed the amount of interfering substance in the neonatal urinary excretion to be quantified; it comprised from 25 to 45% of the total urinary excretion corresponding to between 15 - 34 mg of inulin activity (see page 71).

(ii) CrEDTA. The chromium concentration was measured in 16 control patients and their babies at birth to detect any maternal-foetal difference in chromium concentration. The mean cord chromium concentration was  $4.96 \pm 0.73 \text{ nmol.l}^{-1}$  ( $\pm$  S.E. of mean) and mean maternal chromium concentration  $7.42 \pm 1.23$ . The paired t test showed no significant difference between the maternal and cord serum concentrations ( $p = 0.092$ ) and neither did the Wilcoxon paired rank sum test ( $p = 0.081$ ).

From the control series amniotic fluid specimens were obtained for chromium analysis and all contained  $<2 \text{ nmol.l}^{-1}$  which is the lower limit of the assay. No correction was therefore made for the endogenous chromium content of amniotic fluid.

Blank values for neonatal urinary chromium excretion for the period of urine collection were taken as the chromium content of four of the six urine collections from the group 1 patient-studies. This gave a mean neonatal chromium

excretion of 3.3 nmol over the 48 - 60 hours of urine collection (range 2.1 - 4.2, n = 4).

(iii) Lactulose. There was no lactulose detectable in samples of maternal sera taken before the infusion was begun or in the series of uninfused cord sera.

Blank values for neonatal urinary lactulose excretion over the period of urine collection were estimated from the total lactulose content of five out of the six neonatal urine collections in the first group of patient-studies. This gave a mean value of 19 mg (mean, range 4.2 - 36, n = 5); two of these babies were entirely bottle fed, two fed with a mixture of breast and bottle, and one entirely breast fed. The lowest value of 4.2 was found in the breast fed neonate, and the highest two values in the neonates who were bottle fed, with intermediate values in the neonates fed a combination of breast and bottle milk.

(iv) Mannitol. No mannitol was detected in the pre-infusion samples of maternal sera or cord sera from the series of uninfused pregnancies.

Mannitol was measured by the capillary GC method in pre-infusion maternal urine samples of patient-studies in Group 1, and in samples of neonatal urine of uninfused pregnancies. Three maternal urine samples contained mannitol at concentrations of 0.01, 0.01, and 0.06 mg.ml<sup>-1</sup>, and three neonatal urine samples all contained <0.005 mg.ml<sup>-1</sup> of mannitol.

### 3. Molecular size of CrEDTA.

Four elutions were made on Sephadex G-15 to determine the molecular size of the CrEDTA complex and one of these is illustrated in Fig 18. The resultant  $K_{av}$  values for CrEDTA and the molecular size standards, mannitol, lactulose, and stachyose, together with other relevant data for the latter are presented in Table 6. Plotting the negative logarithm of the value of  $K_{av}$  for stachyose, lactulose, and mannitol against molecular radius gave a straight line that allowed interpolation of the molecular radius for CrEDTA giving a value of 0.64 nm (fig 19).

Table 6. Some properties of the test molecules.

Molecule	Mol. wt.	$a_s^{(2)}$	$K_{av}^{(3)}$
		(Mean $\pm$ S.E.M.)	
Inulin	5200	1.4	-
Stachyose	666	0.72	0.261 $\pm$ 0.011
CrEDTA	387(1)	0.64	0.323 $\pm$ 0.001
Lactulose	342	0.51	0.446 $\pm$ 0.008
Mannitol	182	0.42	0.569 $\pm$ 0.006

(1) As disodium EDTA complexed 1:1 with chromium.

(2) Molecular radius ( $a_s$ ) in nm. from sources quoted in Normand, Olver, Reynolds, & Strang (1971) (inulin, lactulose, & mannitol) and Laurent & Killander (1964) (stachyose), and as derived in the text (CrEDTA).

(3)  $K_{av}$  as measured in the text.

#### 4. Serum profiles.

The profiles of maternal serum values and cord values for patient-studies in group 1 and group 2 are shown in figures 20 - 26.

In the group one studies the cord concentrations for

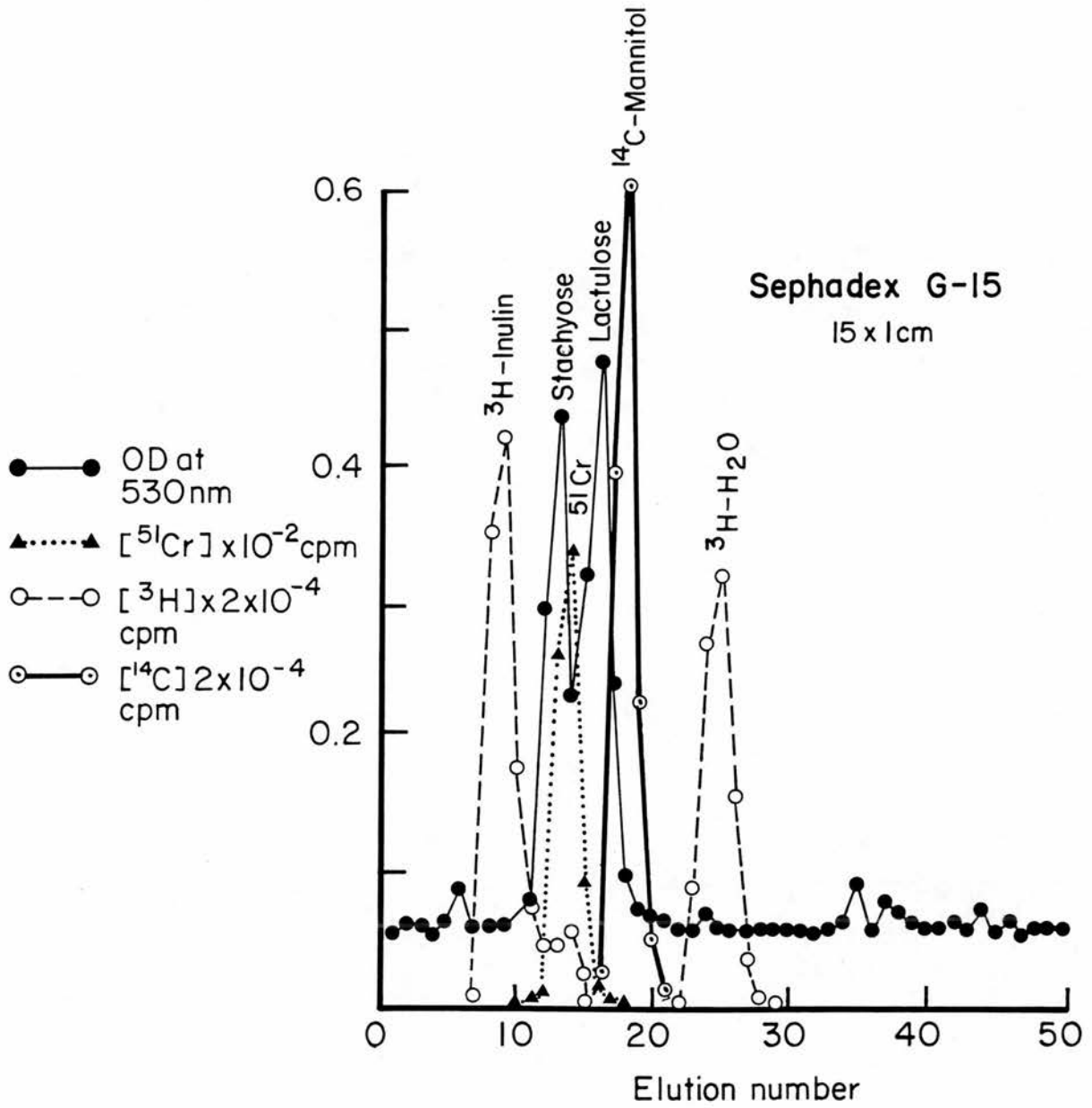


Fig 18. Column chromatography for measurement of the  $K_{av}$  of CrEDTA

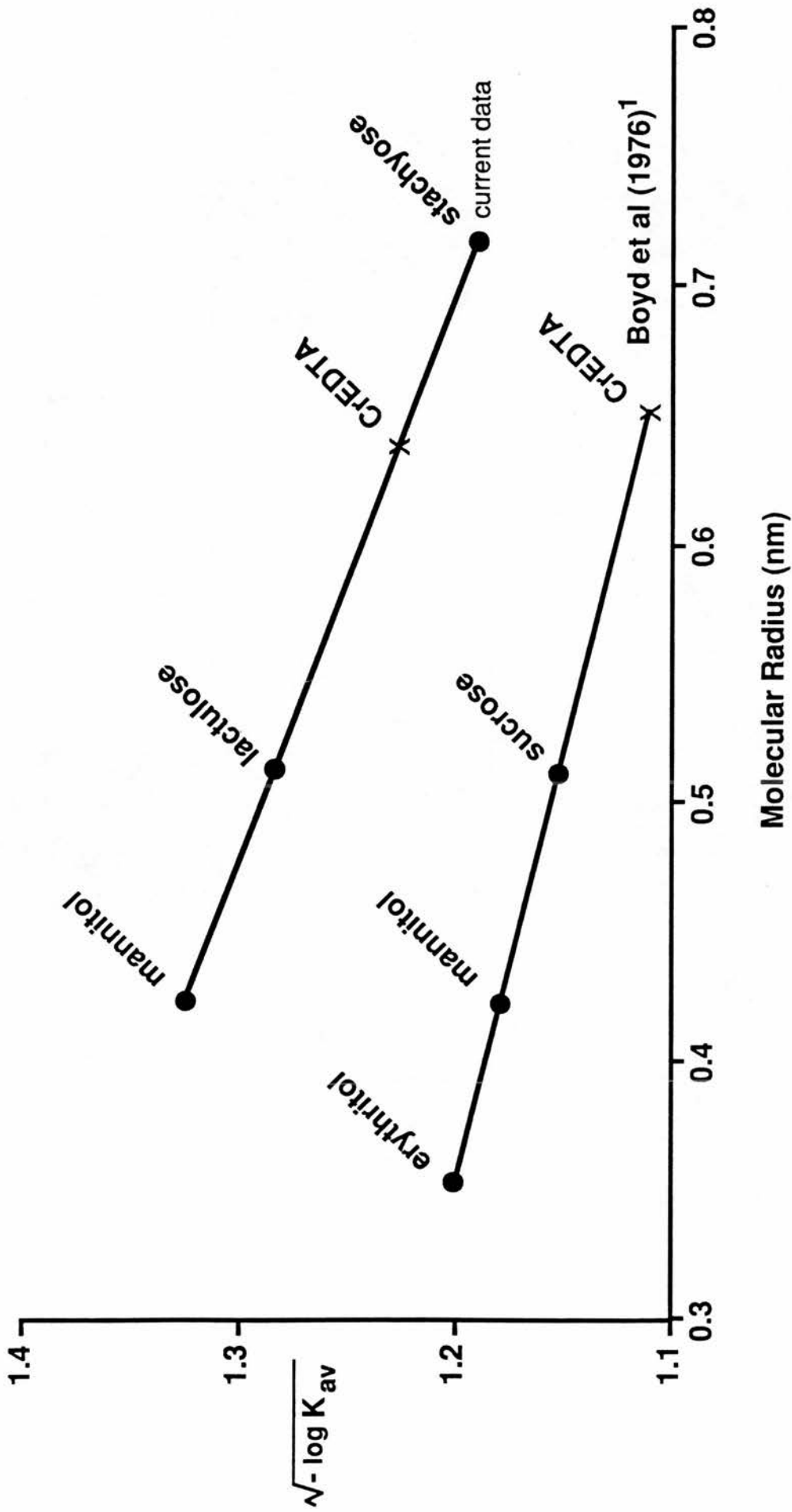


Fig 19. Relationship between  $K_{av}$  and molecular radius allowing the determination of the effective molecular radius of CrEDTA for the current data and that of Boyd et al, (1976). x., is the interpolated datum point for CrEDTA in each study



# Mannitol

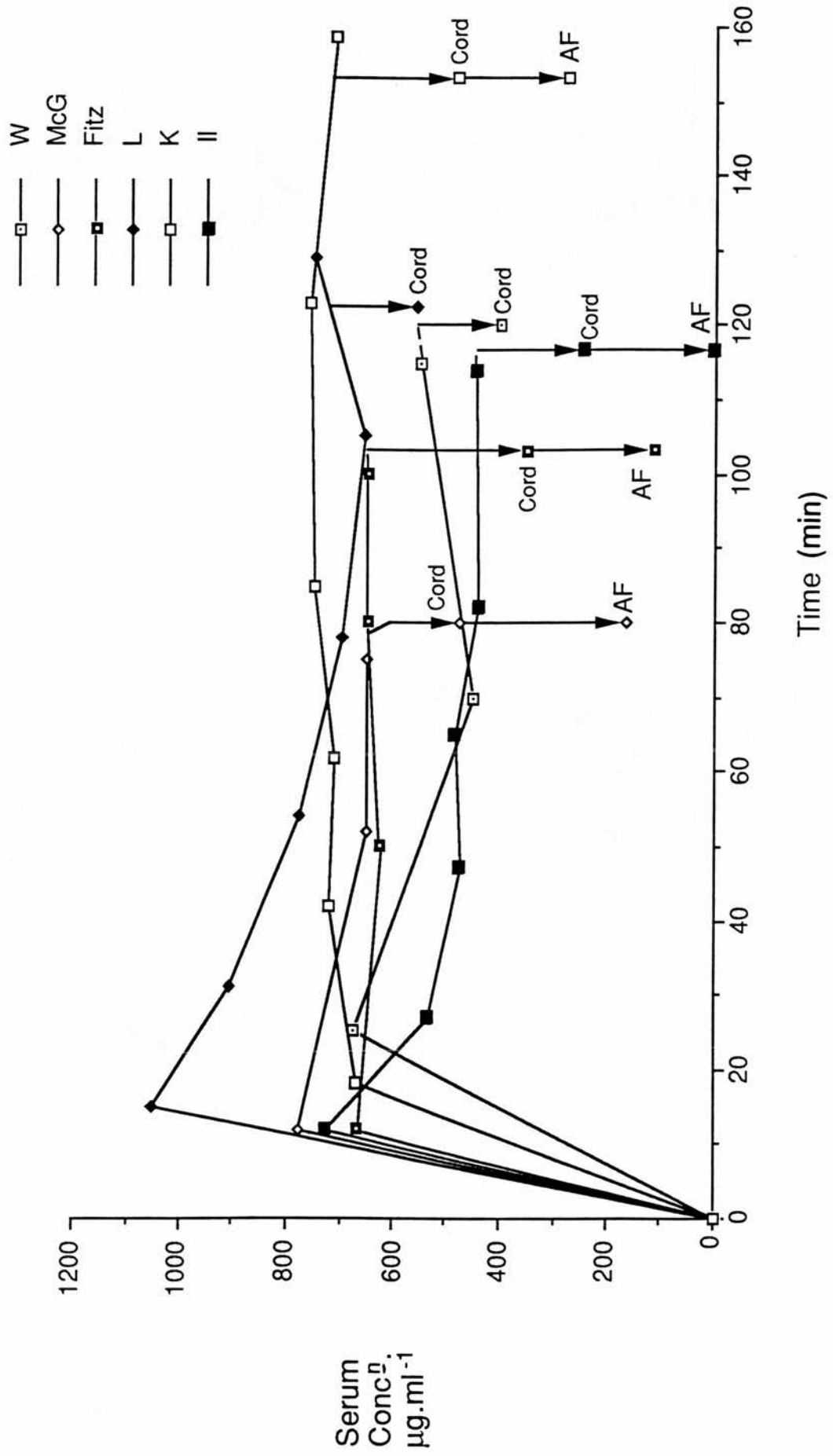


Fig 20. Serum profiles for mannitol in group 1 patient-studies

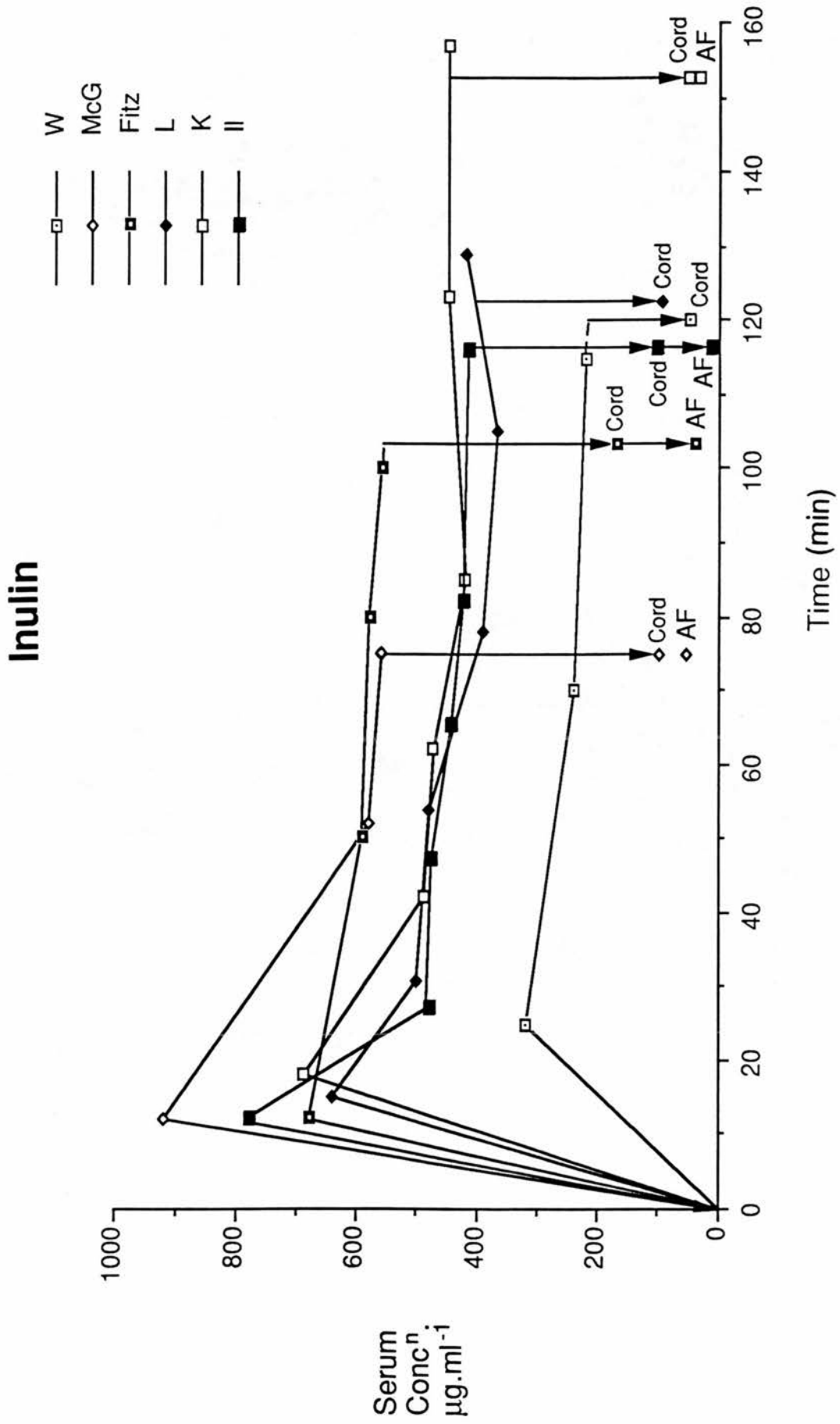


Fig 21. Serum profiles for inulin in group 1 patient-studies

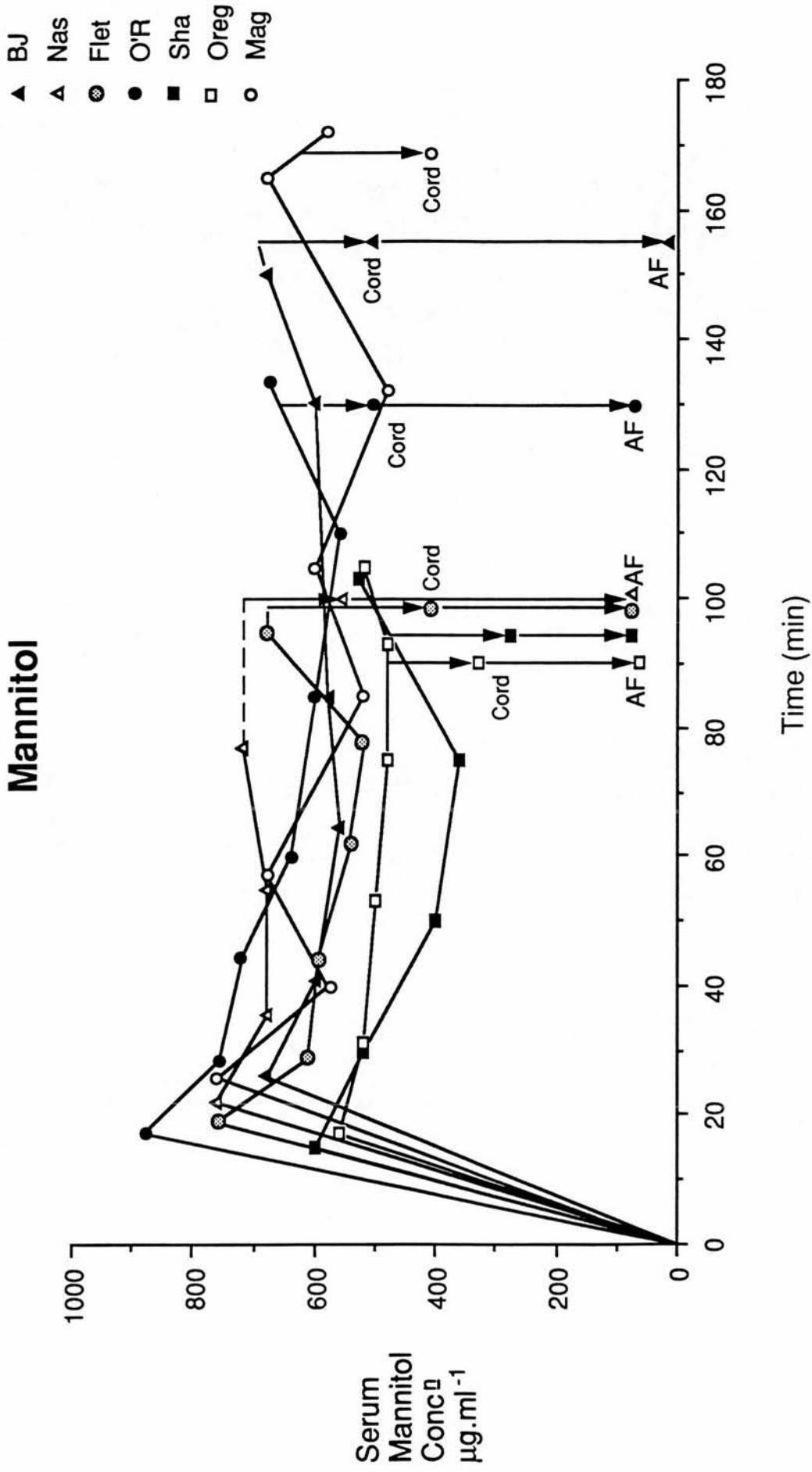


Fig 22. Serum profiles for mannitol in group 2 patient-studies

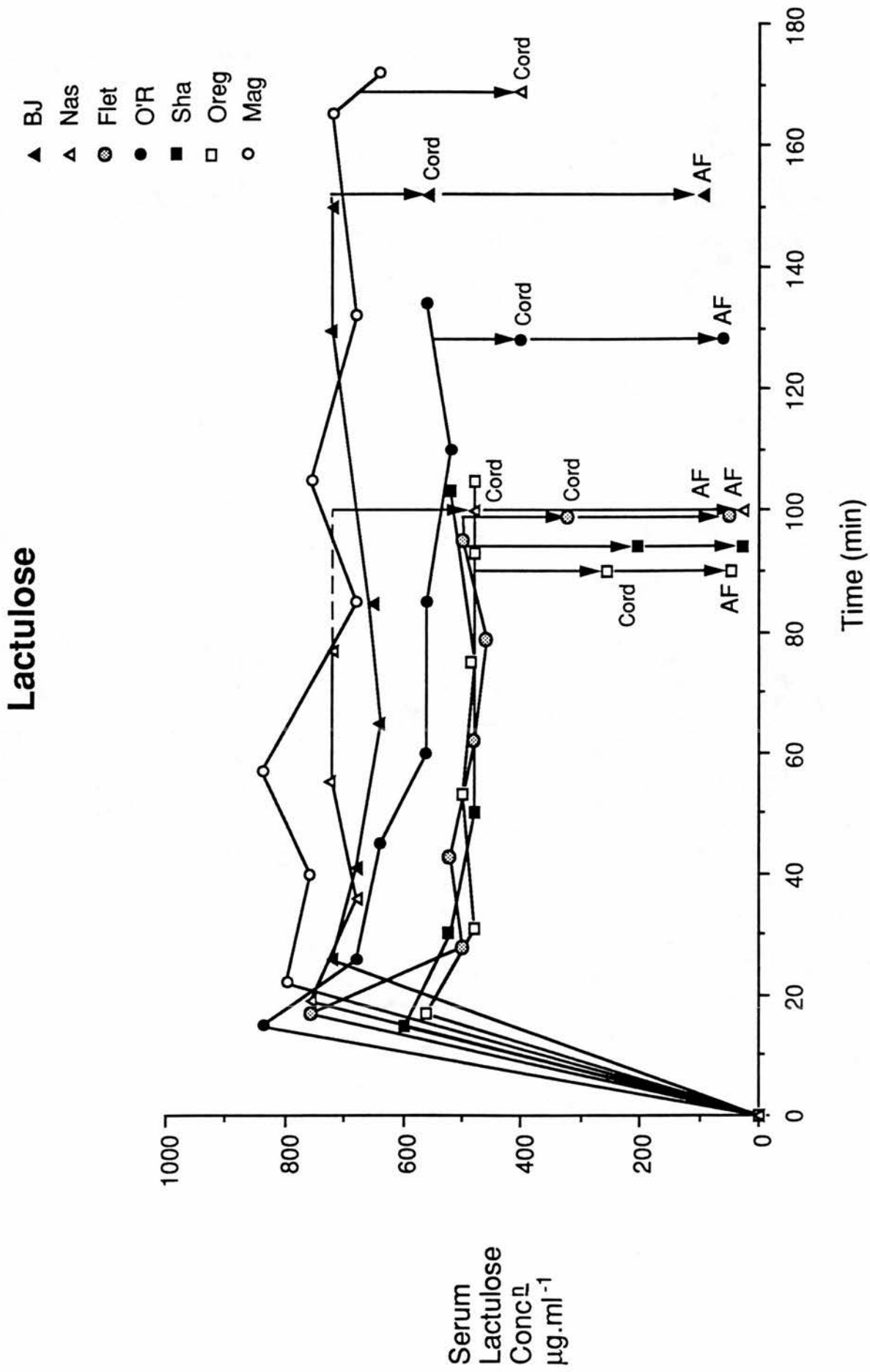


Fig 23. Serum profiles for lactulose in group 2 patient-studies

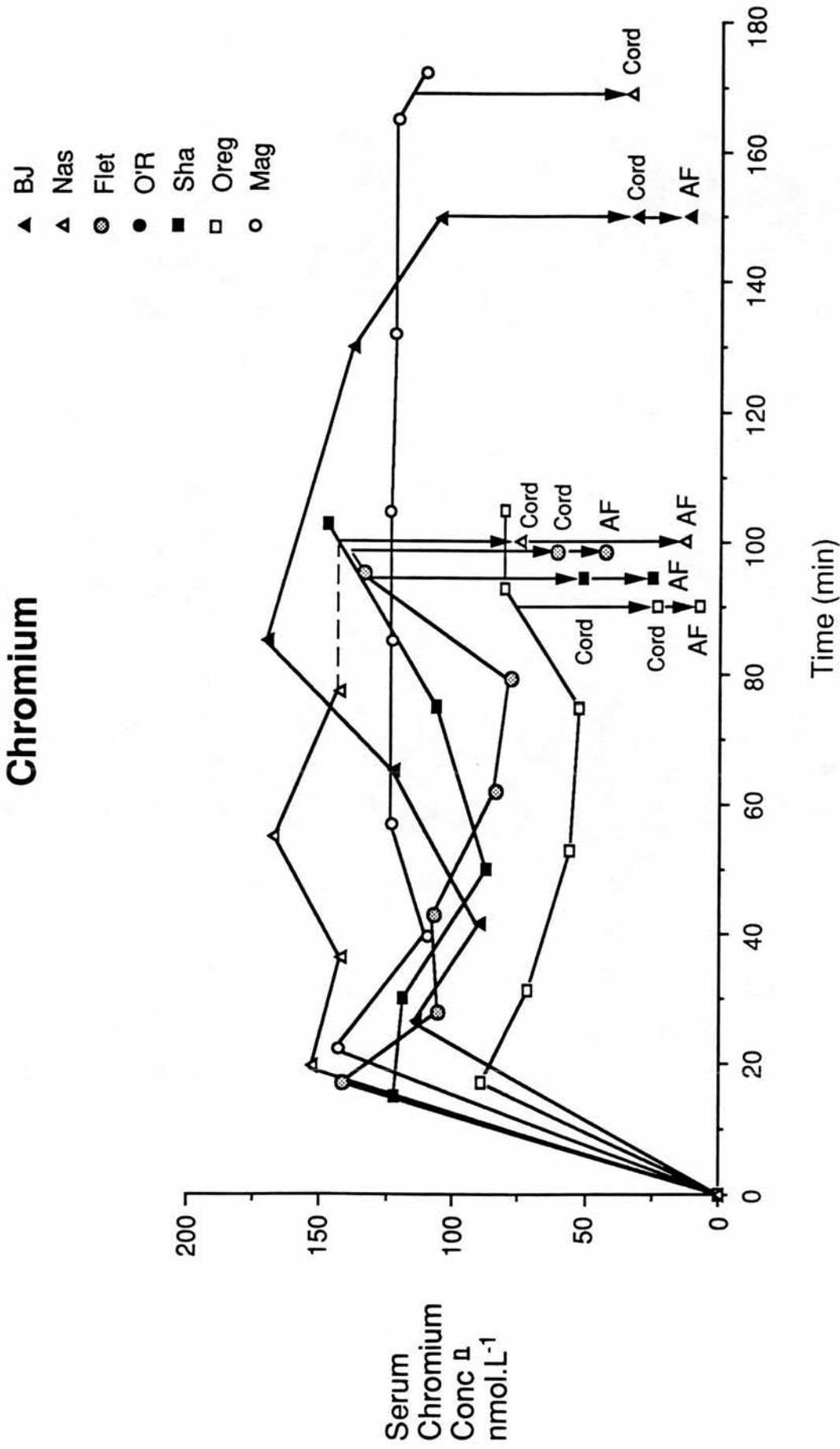


Fig 24. Serum profiles for CrEDTA in group 2 patient-studies

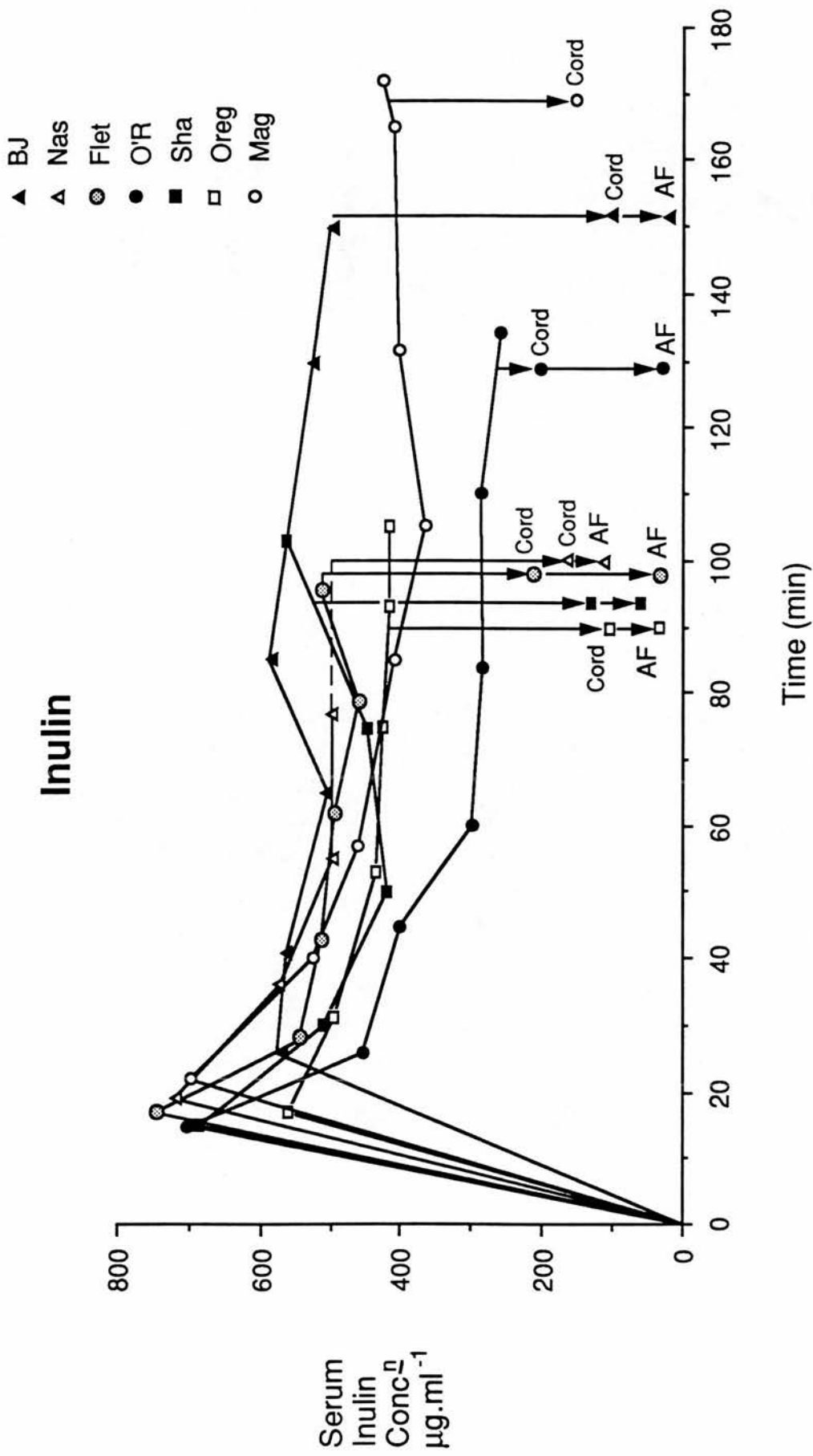


Fig 25. Serum profiles for inulin in group 2 patient-studies

mannitol were  $66 \pm 5\%$  (mean  $\pm$  S.E. of mean) of the corresponding maternal values (at delivery) and  $21 \pm 3\%$  for inulin. The corresponding figures for the patient-studies in group 2 were  $37 \pm 8$  for inulin,  $41 \pm 3$  for CrEDTA,  $62 \pm 5$  for for lactulose, and  $70 \pm 3$  for mannitol. Compartmental analysis (see p 97) indicates that even when the ratio of cord to maternal blood concentration at delivery reaches 0.7 as is the case for mannitol, assuming a linear rise in foetal blood levels will overestimate the maternal-foetal concentration gradient during the infusion by only 11%.

In the group one studies, mean cord levels (for duration of infusion) were  $10 \pm 2\%$  of mean maternal levels ( $\pm$  S.E. of mean) for inulin and  $34 \pm 3\%$  for mannitol. Similarly in the patient-studies in group 2, mean cord levels as a percentage of mean maternal levels were  $17 \pm 3\%$  for inulin,  $24 \pm 3\%$  for CrEDTA,  $32 \pm 3\%$  for lactulose and  $39 \pm 2\%$  for mannitol.

The mean transplacental concentration gradients are listed in Tables 8 and 10.

##### 5. Determined values for placental clearance.

The variables required for the calculation of placental clearance for each of the permeants in both groups of study-patients are listed in Tables 8 and 10: the net flux to the foetus, the transplacental concentration gradient, and the infusion time.

In the group 1 patient-studies the mean mannitol clearance was  $7.3 \pm 0.8 \text{ ml.min}^{-1}$  ( $\pm$  S.E. of mean) and for inulin  $0.86 \pm 0.1$  and in the patient-studies of group 2, 8.7

Table 7. Neonatal details for group 1 patient-studies.

Patient	W	McG	Fitz	L	Il	K
Birth weight of infant (kg)	4.4	3.5	3.0	3.5	3.6	2.9
Placental weight (g)	890	590	490	690	560	560
Sex of infant	M	M	F	F	M	M
Length of infusion (min)	120	80	103	122	116	152
Feeding	Br+Bt	Bt	Br+Bt	Br+Bt	Br	Bt

Br., breast, Bt., bottle

Table 8. Permeability data in group 1 patient-studies.

Patient	W	McG	Fitz	L	Il	K
<u>Mannitol</u>						
Mean conc. gradient ( $\mu\text{g}.\text{ml}^{-1}$ )	285	410	392	458	369	438
Net foetal flux (mg)	286	340	265	339	334	315
Clearance ( $\text{ml}.\text{min}^{-1}$ )	8.4	10.4	6.6	6.1	7.8	4.7
Conc. in amniotic fluid ( $\mu\text{g}.\text{ml}^{-1}$ )	NA	175	125	NA	0	280
<u>Inulin</u>						
Mean conc. gradient ( $\mu\text{g}.\text{ml}^{-1}$ )	213	589	453	384	412	433
Net foetal flux (mg)	32.3	31.8	31.4	40.9	44.8	47.3
Clearance ( $\text{ml}.\text{min}^{-1}$ )	1.26	0.67	0.67	0.87	0.94	0.72
Conc in amniotic fluid ( $\mu\text{g}.\text{ml}^{-1}$ )	NA	65	45	NA	15	45

NA., no fluid available



Table 9. Neonatal details for group 2 patient-studies.

Patient	BJ	Nas	Flet	O'R	Sha	Oreg	Mag
Birth weight of infant (kg)	2.94	3.12	3.0	2.94	3.36	3.22	3.34
Placental weight (g)	630	520	570	600	590	610	540
Sex of infant	F	F	F	F	M	F	M
Length of infusion (min)	152	100	99	129	94	90	169
Feeding	Bt	Bt	Bt	Br	Br	Br	Br

Bt., bottle, Br., breast

Table 10. Permeability data in group 2 patient-studies.

Patient	BJ	Nash	Flet	O'R	Shah	Oreg	Mag
<u>Mannitol</u>							
Mean conc. gradient ( $\mu\text{g.ml}^{-1}$ )	306	363	350	381	281	301	345
Net foetal flux (mg)	393	451	236	215	267	307	423
Clearance ( $\text{ml.min}^{-1}$ )	8.5	12.4	6.8	4.4	10.1	11.3	7.3
Conc. in amniotic fluid ( $\mu\text{g.ml}^{-1}$ )	0	80	80	80	80	68	BS
<u>Lactulose</u>							
Mean conc. gradient ( $\mu\text{g.ml}^{-1}$ )	346	412	321	373	371	333	494
Net foetal flux (mg)	334	371	254	155	167	238	366
Clearance ( $\text{ml.min}^{-1}$ )	6.4	9.0	8.0	3.2	4.8	8.6	4.4
Conc. in amniotic fluid ( $\mu\text{g.ml}^{-1}$ )	100	40	80	40	32	52	BS
<u>CrEDTA</u>							
Mean conc. gradient ( $\text{nmol.l}^{-1}$ )	104	103	61.9	-	82	48	99
Net foetal flux (nmol)	49.6	41.6	31.3	-	18.2	22.9	41
Clearance ( $\text{ml.min}^{-1}$ )	3.1	4.0	5.1	-	2.4	5.3	2.5
Conc. in amniotic fluid ( $\text{nmol.l}^{-1}$ )	12	12	43	-	26	11	BS
<u>Inulin</u>							
Mean conc. gradient ( $\mu\text{g.ml}^{-1}$ )	450	429	392	239	396	378	347
Net foetal flux (mg)	56.3	36	36.3	38.2	25.4	30.9	80
Clearance ( $\text{ml.min}^{-1}$ )	0.82	0.84	0.94	1.24	0.68	0.91	1.4
Conc. in amniotic fluid ( $\mu\text{g.ml}^{-1}$ )	21	116	32	25	58	33	BS

BS., bloodstained

$\pm 1.1$  for mannitol,  $6.3 \pm 0.8$  for lactulose,  $3.7 \pm 0.5$  for CrEDTA, and  $0.98 \pm 0.1$  for inulin. There was no significant difference between the determined clearances for mannitol ( $p > 0.1$ ) and inulin ( $p > 0.1$ ) between patient-study groups 1 and 2. The mean mannitol clearance for all 13 patient-studies was  $8.1 \pm 0.7 \text{ ml.min}^{-1}$  and for inulin  $0.92 \pm 0.07$ , (mean  $\pm$  S.E. of mean).

Expressing the clearance values per unit weight placenta (g), i.e. a permeability surface area product, did not alter the range of values for each permeant, there still remaining an approximately 2 fold difference between the highest and lowest value for inulin and a 3 fold difference for mannitol

#### 6. Correlates of the determined values for placental clearance.

The determined values for placental clearance were tested for correlation against :-

type of infant feeding, breast or bottle. There was no significant difference between the clearances of either breast or bottle fed infants, for mannitol ( $p > 0.5$ ) and inulin ( $p > 0.05$ )  $n = 10$ , for lactulose ( $p > 0.1$ )  $n = 7$ , and CrEDTA ( $p > 0.5$ )  $n = 6$ .

sex of the infant. There was no consistent significant difference between the clearances of study- patients with male or female infants, mannitol ( $p > 0.5$ )  $n = 13$ , lactulose ( $p > 0.05$ )  $n = 7$ , inulin ( $p > 0.5$ )  $n = 13$ , and CrEDTA ( $p = 0.02$ )  $n = 6$  (2 male and

4 female infants).

**anaesthetic, general or epidural.** The type of anaesthetic did not affect the determined placental clearance, mannitol ( $p > 0.5$ )  $n = 13$ , lactulose ( $p > 0.5$ )  $n = 7$ , and inulin ( $p > 0.5$ )  $n = 13$ . Statistics could not be used for the clearance of CrEDTA as 5 of these patient- studies had general anaesthetics (range 2.4-5.1 ml.min<sup>-1</sup>) and only 1 an epidural (5.3 ml.min<sup>-1</sup>).

**maternal age.** The maternal age showed no significant correlation with the determined values for clearance, mannitol  $r = 0.225$  and  $n = 13$  ( $p > 0.1$ ), lactulose  $r = 0.347$  and  $n = 7$  ( $p > 0.1$ ), CrEDTA  $r = -0.510$  and  $n = 6$  ( $p > 0.1$ ), and inulin  $r = 0.146$  and  $n = 13$  ( $p > 0.1$ ).

**placental weight, foetal weight (i.e. birth weight) and combined foetal-placental weight.** There was no correlation between placental weight and the determined values for clearance, mannitol  $r = -0.012$  and  $n = 13$  ( $p > 0.1$ ), lactulose  $r = -0.20$  and  $n = 7$  ( $p > 0.1$ ), CrEDTA  $r = 0.08$  and  $n = 6$  ( $p > 0.1$ ), and inulin  $r = 0.37$  and  $n = 13$  ( $p > 0.1$ ). There was a similar lack of correlation ( $p > 0.1$  in all cases) when foetal and foetal-placental weight were considered.

**infusion time.** Overall there was no consistent correlation between the determined values for clearance and length of infusion, mannitol  $r = -0.56$  and  $n = 13$  ( $p = 0.05$ ), lactulose  $r = -0.55$  and  $n = 7$  ( $p > 0.1$ ), CrEDTA  $r = -0.59$  and  $n = 6$  ( $p > 0.1$ ), and inulin  $r = 0.496$  and  $n = 13$  ( $p > 0.1$ ). The correlation

just achieved significance at the 5% level for mannitol, but not with any of the other three permeants.

When the ratio of the clearances of mannitol to inulin are correlated with time a correlation does appear: slope = 0.098,  $r = -0.674$ ,  $n = 13$  and  $0.02 > p > 0.01$  for the clearance per whole placenta and slope -0.098,  $r = -0.669$ ,  $n = 13$  and  $0.02 > p > 0.01$  for the clearance expressed per unit weight (g) placenta.

**Smoking.** Six of the mothers were smokers and of these 2 stopped smoking during pregnancy leaving 4 who smoked throughout pregnancy. Seven of the mothers were non-smokers. Even by counting those mothers who stopped smoking during pregnancy with those who continued to smoke, the numbers are still too small to be certain of any discernable effect of smoking (Fig 26).

**Molecular size.** The reduction in placental permeability (clearance per gram placenta) with increasing molecular size of each permeant is illustrated in Fig 27 for each of the patient-studies.

## 7. Amniotic fluid.

The concentration of each of the permeants in the amniotic fluid is listed in tables 8 and 10. The effect of including the amniotic content as part of the flux to the foetus is tabulated in Table 13.

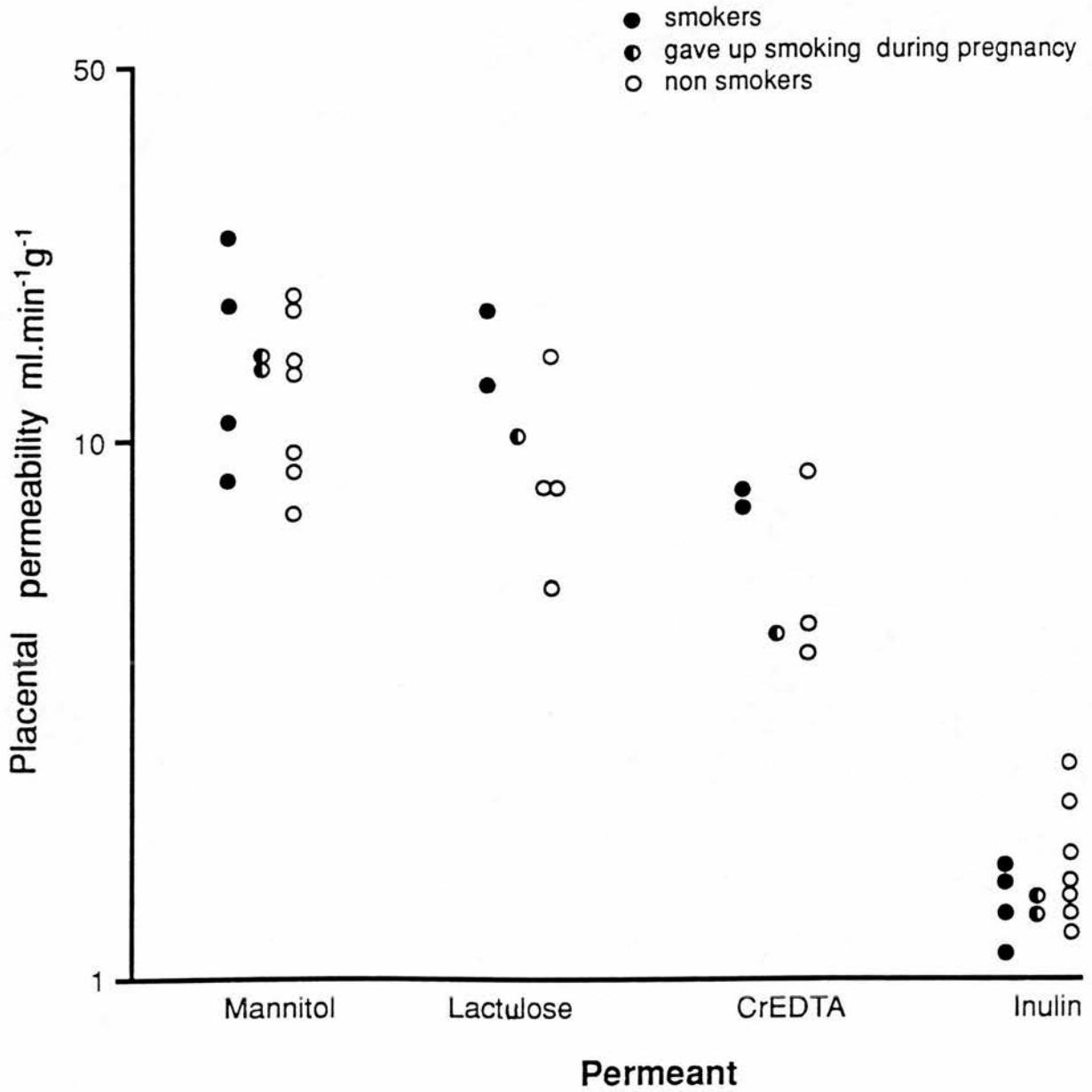


Fig 26. Relationship between placental permeability and smoking habits

## 8. 'in-vivo' versus 'in-vitro'.

Subsequent 'in-vitro' perfusion of the placenta was possible in a total of 9 of the patient-studies; 3 with mannitol alone, 2 with inulin alone, 3 with mannitol and inulin simultaneously, and 1 with mannitol and CrEDTA simultaneously (Landon, Bain, Copas, & Stacey, 1991). The results of the consecutive 'in-vivo' and 'in-vitro' perfusions are compared with the results for all 13 'in-vivo' studies and those from a set of 'in-vitro' perfusions derived by pooling the 'in-vitro' results from the current study with those of a larger, separate, and single permeant per placenta study (Landon, et al, 1991) Fig 28.

The inulin clearances for the consecutive 'in-vivo' and 'in-vitro' studies were  $0.0083 \pm 0.0017$  'in-vitro' (mean  $\pm$  S.E. of mean)  $\text{ml.min.g}^{-1}$  and  $0.0015 \pm 0.0002$  'in-vivo', both  $n = 5$ ; for mannitol 'in-vitro' the clearance was  $0.031 \pm 0.0042$  and  $0.011 \pm 0.0013$  'in-vivo',  $n = 7$ . The ratio of the clearances 'in-vivo' to 'in-vitro' for mannitol and inulin are included in fig 28 and show a much wider spread for inulin than mannitol, inulin range 2.5 to 11.5, mean 6.0; mannitol range 1.4 to 5.2, mean 2.9. Thus 'in-vitro' perfusion increases the measured placental clearances found 'in-vivo' and this increase is greater for the larger molecule inulin. The 3 cases perfused simultaneously both 'in-vivo' and 'in-vitro' with inulin and mannitol and the 1 case similarly perfused with mannitol and CrEDTA (Fig 29), confirmed this effect of 'in-vitro' perfusion of the placental lobule.

The ratios of the clearances 'in-vitro' closely

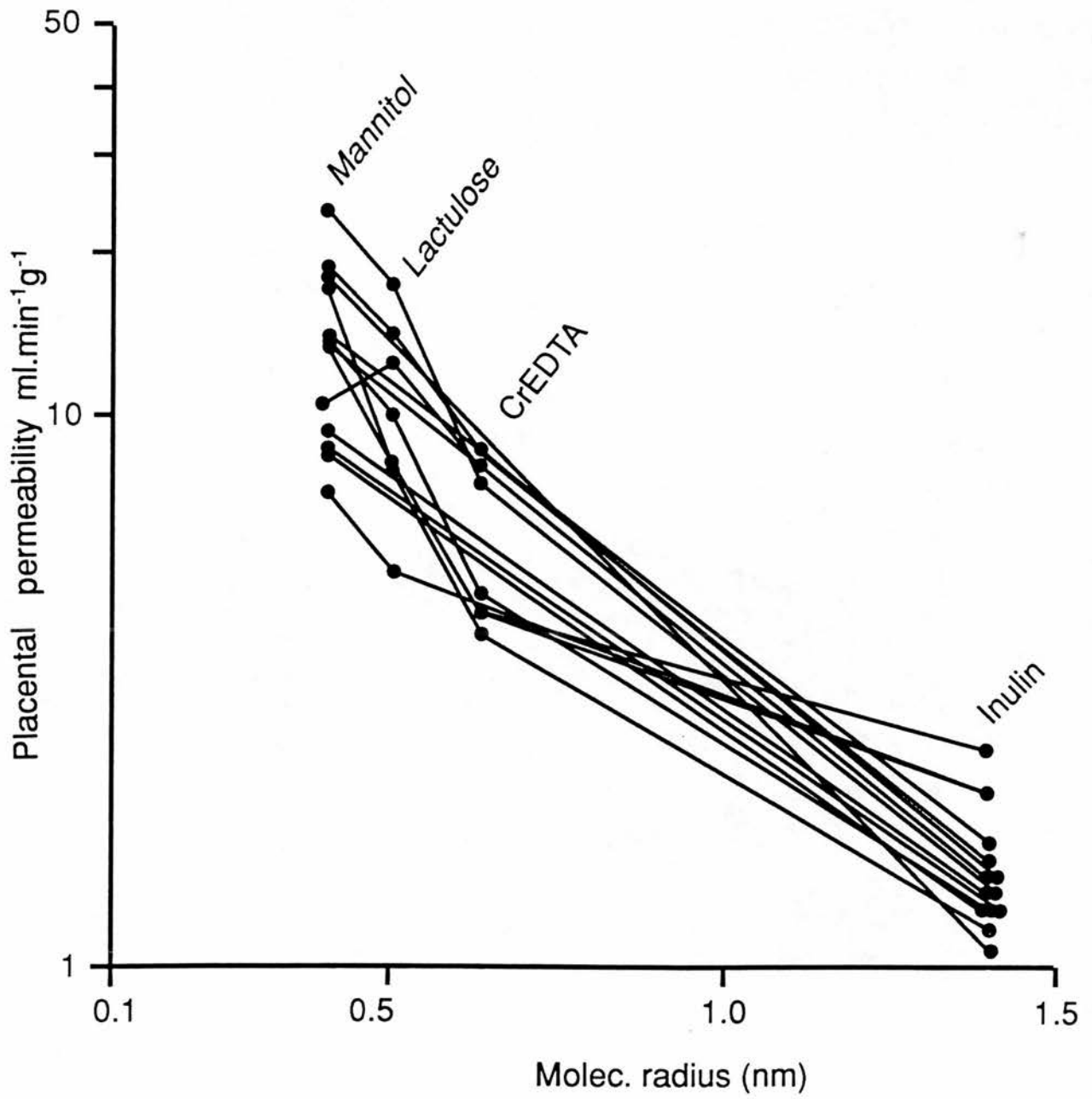


Fig 27. Relationship between placental permeability and permeant molecular radius



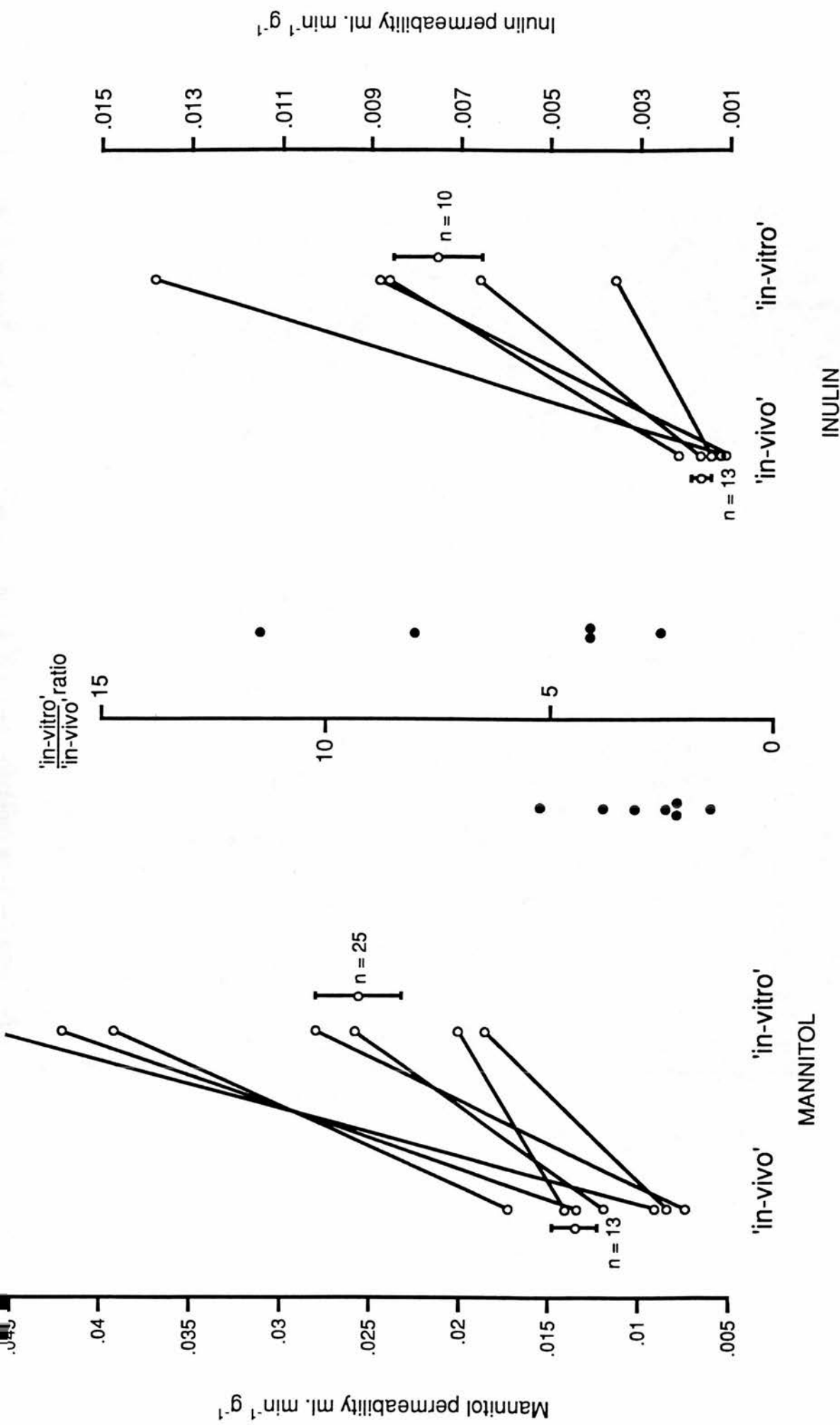


Fig 28. 'In-vivo' and 'in-vitro' placental permeabilities for the sequentially perfused placentas, mannitol  $n=7$ , and inulin  $n=5$ , and the respective 'in-vitro': 'in-vivo' ratio for each placenta studied. For comparison the mean  $\pm$  S.E. of the mean of all 'in-vivo' studies is given ( $n=13$ ), and all 'in-vitro' studies ( $n=25$  for mannitol and  $n=10$  for inulin, (Landon et al, 1991)

approximates to that of the ratios of their coefficients of free diffusion which is not the case for the ratios of the clearances 'in-vivo' (Fig 29). This loss of size selectivity on 'in-vitro' perfusion is demonstrated in Table 11 which uses the three possible ratios and the mean placental clearances from the 13 'in-vivo' patient-studies and the pooled 'in-vitro' data.

#### 9. Volume of distribution.

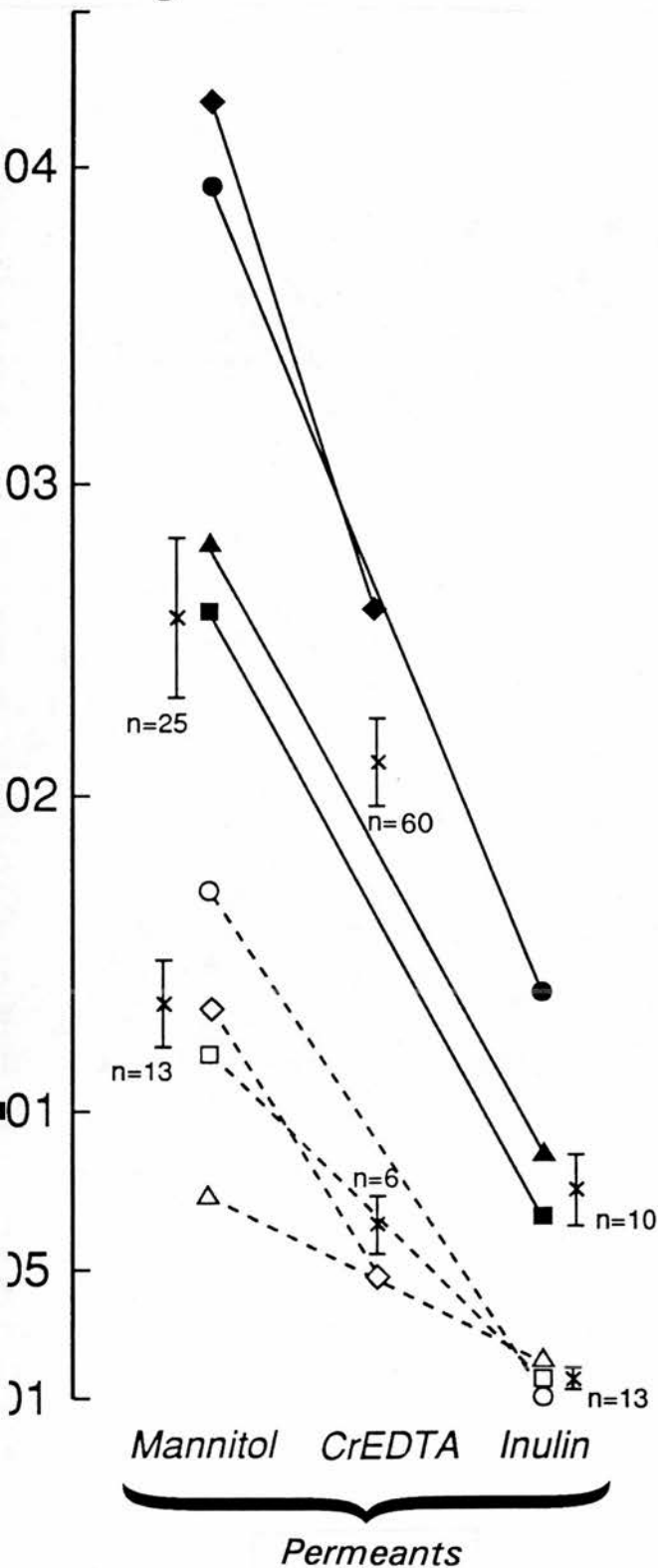
A volume of distribution in the foetus at birth for each permeant can be calculated from the equation,

$$\text{volume of distribution} = \frac{\text{net foetal flux (mg)}}{\text{cord conc. (g.ml}^{-1}\text{)}} \times \frac{1}{\text{birth weight (kg)}}$$

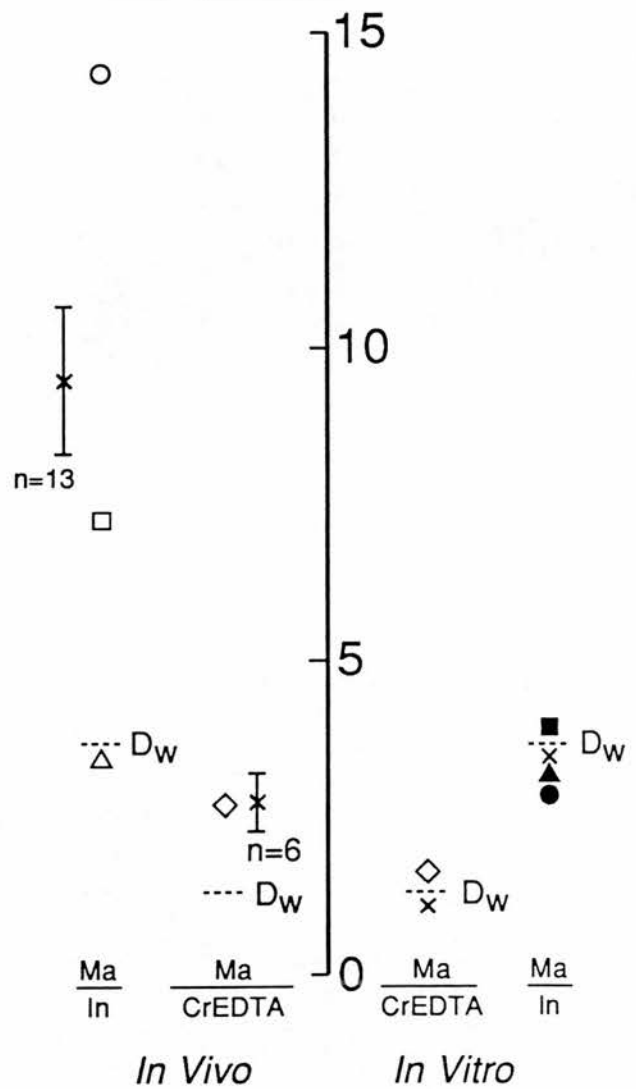
(ml.kg<sup>-1</sup> body weight)

Using the data given in Tables 8 & 10, a volume of distribution of  $240 \pm 20$  ml.kg<sup>-1</sup> body weight (mean  $\pm$  S.E. of mean) was obtained for mannitol,  $240 \pm 20$  for lactulose,  $240 \pm 50$  for CrEDTA, and  $120 \pm 20$  for inulin. There was no significant correlation between the volume of distribution of each permeant and the infusion time: inulin, slope = 0.12,  $r = 0.417$ ,  $n = 13$ ,  $p > 0.1$ ; lactulose, slope = -0.06,  $r = -0.35$ ,  $n = 7$ ,  $p > 0.1$ ; CrEDTA, slope = 0.3,  $r = 0.72$ ,  $n = 6$ ,  $p > 0.1$ ; and mannitol, slope = 0.01,  $r = 0.042$ ,  $n = 13$ ,  $p > 0.1$ .

Placental permeability  
ml.min<sup>-1</sup>.g<sup>-1</sup>



Ratio of  
permeabilities



#### Patient Studies

In Vivo

○ Sha

□ Flet

△ O'R

◇ BJ

In Vitro

● Sha

■ Flet

▲ O'R

◆ BJ

----  $D_w$  Coefficient of diffusion  
in water at 37°C

× means (& S.E. where indicated)  
of the respective 'in vivo' or  
'in vitro' series

Fig 29. Placental permeabilities and ratio of these permeabilities for the 4 patient-studies where 2 permeants were studied sequentially 'in-vivo' & 'in-vitro' in the same placenta. Ma., mannitol; In., inulin. Mean & SE for all 'in-vivo' & 'in-vitro' studies is given where applicable.

Table 11. Ratio of mean 'in-vivo' and 'in-vitro' permeabilities, and the ratio of the coefficients of free diffusion.

Clearance ratio	'In-vivo'	In-vitro <sup>1</sup>	Ratio of coefficients of free diffusion <sup>2</sup>
<u>Mannitol</u>	8.83	3.41	3.65
Inulin	n=13/13	n=25/10	
<u>EDTA</u>	4.21	2.8	2.72
Inulin	n=6/13	n=60/10	
<u>Mannitol</u>	2.1	1.22	1.34
EDTA	n=13/6	n=25/60	

<sup>1</sup>Copas & Landon (1991) - single permeant per placenta

<sup>2</sup>Coefficients of free diffusion in water from Robinson et al (1988)  
n., number of respective placentas studied to obtain the ratio

## Chapter 6.

## Discussion.

## Sources of error.

In measuring placental clearance by the above technique several potential sources of error have to be considered.

(1) The solutes have to be inert, non-metabolizable, to cross epithelia only by passive diffusion, and be excreted wholly and solely by the kidneys. It is generally accepted that all the four permeants used in this study meet these criteria.

(2) The measurement of foetal flux is totally dependant on adequate urine collection. The babies were nursed with their mothers on busy postnatal wards where they received no additional attention because of the study. The urine collection system was therefore devised to require as little attention as possible. The adhesive was effective at keeping the plastic urine bag adherant to the perineum, but immersion in meconium and stool caused it to fail after some time. Breast fed babies with their more frequent bowel actions and more watery stool were worse than bottle fed babies in this regard. During the period of urine collection the babies were visited every 3 - 4 hours during the day to collect the urine passed and to check that the urine bag was still adequately stuck on the perineum. Sometimes the urine bag had to be changed at intervals as short as every 12 hours, and at other

times it would function perfectly well for periods of up to 30 hours. The adequacy of the urine collection could be fairly well assessed by the number of wet nappies. A number of urine collections were obviously lost due to failure of the adhesive, but these were fortunately all after the first 24 hours. As can be seen from fig 12, most of the permeant had been excreted by this time and this source of error should therefore be minimal.

(3) The maternal concentration profile was derived by joining the data points with straight lines. This will tend to under estimate the mean concentration before the first sampling point as the concentration of permeant rises from zero to its peak. This error for the rest of the maternal profile should be much less as the concentration fluctuates much less and the fluctuation is in both directions. The foetal profile was derived by joining the single data point obtained from cord blood at delivery to the origin by a straight line. The actual rise of the foetal serum concentration will however be exponential and compartmental analysis allows measurement of this introduced error which is variable and dependent on the final ratio between foetal and maternal concentrations. Mannitol has the highest ratio of 0.7, as would be expected being the smallest and most permeable molecule, and assuming a linear rise in foetal serum concentrations as opposed to an exponential rise overestimates the mean maternal-foetal concentration gradient by 11%. Assuming a steady-state, this corresponds to an underestimate of 16% in mean foetal concentrations. The corresponding error for a linear rise in foetal

concentrations of the other permeants would be less. These errors in calculating mean maternal and foetal concentrations, both underestimates will tend to be self-cancelling as they are subtracted from each other in the calculation of the clearance.

As the errors introduced by the above simplifications will increase with time causing a progressive overestimate of clearance with time, the lack of any significant correlation between the clearance values for each permeant and the length of infusion supports the contention that the errors introduced by these mathematical assumptions is insignificant compared to the individual variation in placental clearance and the errors inherent in all the measurements. The correlation between the ratio of the clearances of mannitol to inulin and the length of the infusion could be due to many factors singly, or in combination. The correlation may be due to chance as it only achieved significance at the 2% level. The error in the assumptions obviously increases with time and this will be most marked with the most permeable permeant, namely mannitol. It is interesting therefore that mannitol does show a correlation with the length of infusion that only just reaches the 5% level of significance.

(4) The significance of the amount of permeant in the amniotic fluid and whether it should be included as part of the net foetal flux is contentious. If the permeant in the amniotic fluid arises solely from the placenta and foetal urinary excretion, then it should properly be included in the calculation of placental clearance. If, on the other hand, it arises from direct transfer across the foetal membranes

independent of the foetal-placental unit, then it should not be included in the calculations. There is unfortunately no precedent in the literature to clarify which approach is correct. Willis et al (1986) from Oregon University measured the permeability of the placenta to cyanocobalamin and excluded the amniotic fluid content in the measurement of flux to the foetus on the grounds that its content was negligible, 320 ng assuming an amniotic fluid volume of 1 litre compared with a foetal content of 4.5  $\mu$ g. In a subsequent publication from the same group however, Thornburg et al (1989) included the amniotic fluid content of inulin as part of the measured flux to the foetus and therefore included it in the calculation of the placental clearance of inulin. Indeed, the mean inulin content of the amniotic fluid was twice that of the foetus while in one case it was nine times the foetal content. Unfortunately inulin cannot be directly compared with cyanocobalamin as the latter is protein bound in the plasma and in tissues and only when this capacity is exceeded is there significant renal clearance.

There is already evidence from the literature that direct transfer occurs across the extra-placental foetal membranes from maternal blood directly into the amniotic fluid bypassing the foetus. In the original report by Basso et al (1977) demonstrating that mannitol was mostly entering the amniotic fluid through foetal micturition, one pregnancy with a dead foetus was described. In that pregnancy the levels of mannitol in amniotic fluid rose only slowly and did not exceed those in maternal blood, indicating that some mannitol was being transferred directly from the maternal vascular



space to the amniotic fluid bypassing the placenta. Super, Fennell, Schwartz & Boyd (1986) have recently reported that the usual form of non-specific cholinesterase in amniotic fluid is of maternal not foetal origin. This contrasts with the specific acetylcholinesterase which is of foetal origin and used in the diagnosis of neural tube defects (Seller & Cole, 1980). Johnson, Umansky, Alper, et al (1974) analysed the correlation between the ratio of the concentration of several proteins in amniotic fluid and maternal blood with their molecular weight and compared this with the correlation of the ratio between the concentrations in amniotic fluid and cord blood with molecular weight. The correlation was significantly closer ( $r=-0.925$ ,  $p<0.0005$ ) and there was much less scatter about the least-squares regression line with the amniotic fluid : maternal blood ratio, than amniotic fluid : cord blood ratio ( $r=-0.6767$ ,  $p=0.02$ ) over a molecular weight range 40,000 to 800,000. This indicates that amniotic fluid proteins in general are primarily a simple filtrate of maternal plasma. The concordance with maternal genotype for the two proteins examined (MW 44,000 and 51,000) rather than foetal genotype provides confirmation of the maternal origin of these proteins and indicates that the foetal contribution to the amniotic fluid is less than between 2 - 10 percent, the lower limit of sensitivity of the technique, certainly for these two proteins and most probably for proteins of higher molecular weight. The passive transfer of plasma proteins indicates a route that would be available for the passive transfer of the much smaller inulin molecule.

The amniotic fluid content of the permeants depends not

only on their measured concentration, but on the measured amniotic fluid volume as well. Measurements of amniotic fluid volume are notoriously difficult to make in routine clinical practice.

In the current study the origin of the permeant in the amniotic fluid might be checked in two ways. Firstly, it is possible to determine whether the amniotic fluid content could be accounted for by the estimated foetal urine production rate. Such a back calculation was possible only for study K, however, because the individual urines were only assayed in the patient-studies in Group 1, amniotic fluid was not obtained from every patient, and a urine sample at birth was available from only a few patient-studies (Table 11). In patient-study K the first urine at birth contained inulin at  $0.375 \text{ mg.ml}^{-1}$  and mannitol at  $1.8 \text{ mg.ml}^{-1}$ . Assuming an amniotic volume of 1 litre (Lind 1981), this corresponds to a minimum foetal urine production of  $47 \text{ ml.hr}^{-1}$  for inulin and  $61 \text{ ml.hr}^{-1}$  for mannitol. The data of Thornberg et al, (1988) allows a similar analysis in their patient number 3 with a quoted neonatal urinary inulin concentration of  $147 \text{ } \mu\text{g.ml}^{-1}$  in the presumed first urine specimen passed, giving a foetal urine production rate of 600 ml over a mean infusion period of 3 hours ( $\pm 1.75$ , the SD). These figures compare with the published fetal urine production rate of  $23 - 28 \text{ ml.hr}^{-1}$  for term infants (Campbell, Wladimiroff, & Dewhurst, 1973) found by serial measurements of bladder volume by ultrasound. This is strong evidence that fetal urine is unlikely to be the sole source of the permeant in amniotic fluid.

A second possible indication of the origin of the

permeants in amniotic fluid would be their ratios (cf Johnson et al, 1974). If foetal in origin, the ratio of mannitol to inulin concentration in amniotic fluid should resemble the ratio in either cord blood or the neonatal urine at birth. If maternal in origin, the ratio should resemble that found in maternal blood as certainly the extra-placental membranes permit the passage of large protein molecules. Unfortunately the data (Table 12) are insufficient to allow a definitive conclusion.

It is impossible to distinguish between, and measure separately, that proportion of permeant derived from foetal micturition and that derived from other sources. If the amniotic fluid content is included as part of the foetal flux then the clearances will increase as illustrated in Table 13 which assumes an amniotic fluid volume of 1 litre. This increase shows marked variability between patient-studies and between the different permeants used. In the group 2 patient-studies, including the amniotic fluid content of permeant in the net flux to the foetus increases the clearance for inulin by 150%, that for CrEDTA by 60%, and that for lactulose and mannitol by 25%. The relatively greater amniotic fluid content relative to foetal content for inulin and to a much lesser extent for CrEDTA compared with mannitol and lactulose, does not fit with either a foetal or extra-placental origin. It is more likely due to the insensitivity of the inulin assay at the low concentrations found in amniotic fluid (at the limit of sensitivity of the assay a small change in optical density can double the inulin content which is magnified by the correction for the 1:10 dilution) and the

Table 12. Ratio of clearances and permeant concentrations in amniotic fluid and other body fluids.

Patient- study	$[m_a]:[i_n]$ ratio in AF	Placental clearance $m_a:i_n$	Urinary $[m_a]:[i_n]$ at birth	$[m_a]:[i_n]$ cord blood	$[m_a]:[i_n]$ maternal blood
K	6.2	6.6	4.9	9.0	1.5
L	NA	7.0	5.8	5.5	1.7
Fitz	2.8	9.8	NA	2.5	1.0
McG	2.7	15.5	NA	4.5	1.0
Il	0	8.3	NA	2.0	1.0

NA., not available; AF., amniotic fluid.  
 $m_a$ , mannitol;  $i_n$ , inulin; [ ], concentration.

Table 13. Placental clearances excluding and including amniotic fluid content.

Patient study	W	McG	Fitz	L	Il	K	BJ	Nash	Flet	O'R	Shah	Oreg	Mag
<u>Clearance</u> (ml.min <sup>-1</sup> )													
Mannitol													
excl AF	8.4	10.4	6.6	6.1	7.8	4.7	8.5	12.4	6.8	4.4	10.1	11.3	7.3
incl AF	NA	15.7	9.7	NA	7.8	8.9	8.5	14.6	9.1	6.0	13.1	13.8	B.S
Lactulose													
excl AF	-	-	-	-	-	-	6.7	9.5	8.6	3.6	5.3	8.6	4.6
incl AF	-	-	-	-	-	-	8.6	10.4	11.1	4.5	6.3	10.3	B.S
CrEDTA													
excl AF	-	-	-	-	-	-	3.1	4.0	5.1	IS	2.4	5.3	2.4
incl AF	-	-	-	-	-	-	3.9	5.2	12.1	IS	5.7	7.9	B.S
Inulin													
excl AF	1.26	0.67	0.67	0.87	0.94	0.72	0.82	0.84	0.94	1.24	0.68	0.91	1.4
incl AF	NA	2.05	1.64	-	1.25	1.40	1.13	3.54	1.76	2.05	2.24	1.88	B.S

excl AF., excluding amniotic fluid  
incl AF., including amniotic fluid  
NA., amniotic fluid not obtained  
BS., blood stained  
IS., insufficient sample

presence of a variable but more equal background level. Both these factors would apply to a much lesser extent to the assay for chromium.

With all the above uncertainties over the amniotic fluid content of permeant, it is probably more realistic at the present time to express the results excluding it.

(5) Validity of the assumed model. The calculation of placental clearance assumes a two compartment model, namely maternal and foetal, with equilibration across the placental membrane with no additional compartments. The maternal infusion achieves near steady-state levels in the maternal compartment, but there remains the possibility of a slowly equilibrating compartment in the foetus. The lack of any significant correlation of clearance with time indicates the absence of a significant additional compartment in the foetus, as this would lead to a progressive underestimate of the net foetal flux and therefore a fall in clearance with time. A two compartment model therefore appears to apply for the length of infusion period used.

The significance of the errors inherent in the method has to be seen in the context of a 2 - 3 fold variation in placental clearance between individuals, that is likely to dwarf any experimental error.

#### Comparison with other available data.

Molecular size of CrEDTA. The data of Boyd et al (1976) can be used to derive a molecular radius for CrEDTA when the negative logarithms of the  $K_{av}$  values are plotted against the molecular radius. This gives a straight line and a

molecular radius for CrEDTA of 0.65 nm (Fig 19), almost identical to the value found in the present study of 0.64 nm for the molecular radius of CrEDTA.

Both the human placenta 'in-vivo' and Sephadex gel handle CrEDTA as if it were a larger molecule than suggested by the molecular weight. It is well recognised that filtration through Sephadex can be affected by the charge carried on a small molecule, and as CrEDTA is a weakly charged anion, it is possible that the placenta, like Sephadex gel, is discriminating against the charge on the CrEDTA molecule. An alternative explanation for the discrepancy in apparent size molecular may relate to a hydration shell around the CrEDTA molecule, though it is interesting that Bradbury (Hedley & Bradbury, 1980) suggested that the guinea pig placenta may discriminate against molecular charge.

'In-vivo' human studies. The only directly comparable data on 'in-vivo' placental clearances in man are those of Willis et al (1986) using cyanocobalamin and Thornburg et al (1988) who used inulin. Both these studies originated from the Department of Physiology in Oregon and used parenteral administration of a single permeant to achieve adequate maternal levels; in the case of cyanocobalamin a single intramuscular injection was used, while inulin was administered as a bolus followed by a continuous infusion. The same mathematical principles as in the current study were employed to calculate a maternal-foetal gradient. Compared to the present study, the study period as quoted was shorter for cyanocobalamin, average 42 min, range 27-110 but longer for inulin,  $176 \pm 107$  min (mean  $\pm$  S.D.).



These two studies differed in three important methodological aspects from the current study.

Firstly, the maternal-foetal flux, equal to the accumulation by the foetus, was calculated in both studies not from the subsequent neonatal excretion but from a cord blood value multiplied by a putative factor for the volume of distribution derived from parallel studies on guinea pigs. This effectively replaces errors in urine collection which are quantifiable with the errors implicit in applying a factor derived in another species without validation. The guinea pig placenta differs from the human placenta in being a much smaller proportion of the foetal weight than the human, 7% in Willis et al's study (1986) as opposed to the 18% in the current study. Willis et al (1986) measured the volume of distribution factor as  $280 \text{ ml.kg}^{-1}$  body weight plus placental weight for cyanocobalamin corresponding to  $300 \text{ ml.kg}^{-1}$  body weight alone (mean placental weight 7% of foetal weight) and Thornburg et al (1988) measured a corresponding figure of  $180 \text{ ml.kg}^{-1}$  body weight for the volume of distribution of inulin. These figures compare with the volumes of distribution from the current study of  $240 \text{ ml.kg}^{-1}$  body weight for CrEDTA, the nearest permeant in size to cyanocobalamin, and  $120 \text{ ml.kg}^{-1}$  for inulin. All these figures compare with other estimates of extracellular fluid space in healthy term neonates of approximately  $380 \text{ ml.kg}^{-1}$  (Cassady, 1970). This would suggest that none of the permeants was evenly distributed throughout the extracellular fluid space during the period of infusion.

Secondly, cobalamin is a normal tissue component that is



vital for normal tissue metabolism and for which there are specific carrier mediated transport mechanisms and metabolic pathways. Although Willis et al (1986) attempted to saturate the endogenous tissue mechanisms by using large doses of cyanocobalamin such that diffusional transfer would predominate, there remains doubt as to the contribution of carrier mediated transfer and the extent to which metabolism may occur. Cyanocobalamin transfer does not therefore solely measure passive diffusion.

Thirdly, the amniotic fluid content was included as part of the net foetal flux when the inulin clearance was measured but excluded when the clearance of cyanocobalamin was determined.

Considering the differences outlined above, the values for the placental clearances of cyanocobalamin and inulin measured in the studies by Willis et al (1986) and Thornburg et al (1988) are of the same order of magnitude as those found in this study. For cyanocobalamin, a value of  $9.6 \text{ ml.min}^{-1}$  was measured, just over twice the value of slightly less than  $4 \text{ ml.min}^{-1}$  predicted by the current data, this being the clearance value for CrEDTA, the nearest permeant in the current series. For inulin, excluding the amniotic fluid content, Thornburg et al (1988) found a mean inulin clearance of  $1.4 \text{ ml.min}^{-1}$ , quite close to the figure of  $0.9 \text{ ml.min}^{-1}$  for all 13 patient-studies in the present study; the difference can be largely accounted for by the different volumes of distribution between the two studies.

Faber and Thornburg (1983) have recalculated the data of Flexner et al (1948) to give a permeability surface area

product for sodium in the human of  $3.4 \times 10^{-2} \text{ ml.min}^{-1}.\text{g}^{-1}$ . Sodium, however is not an inert permeant and the data is not directly comparable with that of the current study.

'In-vitro' human studies. The current data for 'in-vivo' placental clearances expressed per unit weight of placenta, i.e.  $\text{ml.min}^{-1}.\text{g}^{-1}$ , are compared with the results of 'in-vitro' perfusion of the placental lobule from Landon et al (1991) and Schneider, Sodha, Proegler, and Young (1985) in Table 14.

Table 14 Comparison with 'in-vitro' perfusion data.

Permeant (MW)	Current data ( $\text{ml.min}^{-1}.\text{g}^{-1}$ )	Proegler et al <sup>1</sup> ( $\text{ml.min}^{-1}.\text{g}^{-1}$ )	Landon et al <sup>2</sup> ( $\text{ml.min}^{-1}.\text{g}^{-1}$ )
Mannitol (182)	$13.5 \times 10^{-3}$	-	$25.6 \times 10^{-3}$
L-Glucose (182)	-	$23 \times 10^{-3}$	-
Lactulose (342)	$10.8 \times 10^{-3}$	-	-
Sucrose (342)	-	$19 \times 10^{-3}$	-
CrEDTA (387)	$6.33 \times 10^{-3}$	-	$21.0 \times 10^{-3}$
Inulin (5200)	$1.53 \times 10^{-3}$	$6 \times 10^{-3}$	$7.5 \times 10^{-3}$

<sup>1</sup> Data from Schneider et al (1985)

<sup>2</sup> Data from Landon et al (1991)

The comparison in Table 14 indicates the close agreement between the results of 'in-vitro' perfusion of the placental lobule by Landon et al (1991) at the centre where the current work was undertaken and Schneider et al (1985) but with an obvious difference between both these 'in-vitro' studies and the present 'in-vivo' studies. Not only does the placental lobule perfused 'in-vitro' have a greater

clearance than that measured 'in-vivo', but the difference between the 'in vitro' and 'in-vivo' data is greater with inulin (approximately 5 times) than with mannitol (approximately 2 times). The ratio of the clearances in the current study (Figs 28 & 29) clearly indicates that while the 'in-vivo' data indicates molecular size restriction by the placenta (clearance ratios greater than corresponding ratios of coefficients of free diffusion), 'in-vitro' perfusion reveals a placental membrane that shows no molecular size discrimination between mannitol and inulin (clearance ratios equal corresponding ratios of coefficients of free diffusion).

The reason for the discrepancy between the clearances measured 'in-vivo' and 'in-vitro' is not yet clear. 'In-vitro' perfusion of the placental lobule does not duplicate the blood flows found 'in-vivo', which in any case are not yet defined, and this is evidenced by 2 observations. Firstly, 'in-vitro' perfusion attempted through the umbilical artery is unsuccessful due to the inordinately high pressures required to generate flow and is only successful when a foetal artery and vein on the periphery of a placental lobule are used. The foetal vasculature is not therefore behaving as it does 'in-vivo'. Secondly, doubling the number of cannulae perfusing the intervillous space (i.e. the maternal side) but keeping the the overall perfusion rate rate unchanged (i.e. flow per cannula halved), causes a significant drop in measured placental clearance (Landon MJ, personal communication).

While changes in flow rate 'in-vitro' could increase the

measured clearances by increasing placental transfer in poorly perfused areas of the placenta (increased efficiency of placental transfer), there should not be any effect on the molecular sieving properties of the placental membrane, that is, there should be no effect on the mannitol to inulin clearance ratio which there clearly is. This loss of size restriction when the placenta is perfused 'in-vitro' indicates that more than flow has been altered because size discrimination is a fundamental characteristic of the placental membrane itself. The technique of 'in-vitro' perfusion of the placental lobule is rarely successful with blood as perfusate and physiological replacements for blood are routinely used instead; if the perfusate contains fibrinogen, fibrin is deposited in the placenta and if red cells are used in the perfusate they must be washed. The effect of 'in-vitro' perfusion therefore very much resembles the 'protein effect' observed when albumin is washed out of capillaries during physiological studies of capillary permeability and which has been used as evidence for the fibre matrix theory of capillary permeability (Levick, 1991).

While attempts to assess the metabolic and structural viability of the 'in-vitro' perfused placental lobule have demonstrated the preparation to be intact within the limits of the parameters assessed, no tests of any transfer function were carried out (Illsley, Aarnoudse, Penfold et al, 1984). The four sequential studies (Fig 29) avoid the difficulty of trying to distinguish any effect of 'in-vitro' perfusion from a two to three fold variation in placental

permeability between individuals. They confirm the effect of 'in-vitro' perfusion as increasing the measured values for placental clearance and as eliminating size discrimination by the placental membrane. Although one of the three placentas that was perfused both 'in-vivo' and 'in-vitro' had a mannitol to inulin clearance ratio that equalled the ratio of the coefficients of free diffusion (patient-study, O'R), this was the only patient-study to demonstrate such a low ratio (high inulin and low mannitol clearance) and was not distinguishable from the other patient-studies in any methodological or clinical characteristic.

Hedley & Bradbury (1980) have already demonstrated that 'in-situ' perfusion of the guinea pig placenta using a physiological perfusate increases the measured placental permeability, by 84% for erythritol (MW 122), 234% for CrEDTA, and 263% for FITC dextran (MW 3000). This is very similar to the situation found in the human placenta by the current study and provides a precedent for significant distortion of placental transfer by the technique of 'in-vitro' perfusion. Robinson et al (1988) however, compared 'in-vivo' and 'in-situ' perfusion of the rat placenta and were unable to show any significant difference in placental permeability between these two situations; interestingly, the standard error of the mean measured 'in-situ' was greater than measured 'in-vivo' and this was quite marked for the permeants mannitol and inulin. The reason for the resistance of the rat placenta to perfusional change in permeability is unclear at present.

Animal studies. The comparable animal data tabulated in

Table 1 is presented graphically with the current placental permeability data again expressed per unit weight of placenta (Fig 30). This shows that the current data most closely resemble those of the guinea pig, rat, and rabbit. These animals have placental permeabilities all within an order of magnitude of the human, with a similar overall decrease in permeability with increasing molecular size. The different profile of the rabbit and one set of data for the guinea pig is due entirely due to one datum point in each study. The contrast of the profiles of these animals and that found in the present study for the human with that of the sheep is striking.

Figure 30 also emphasises two other features of placental permeability in man and the rabbit, guinea pig, and rat. Firstly, there is an approximately fifteen fold variation between these species in the permeability to any given permeant in the molecular weight range studied and secondly, this compares with a 10 fold variation in permeability with molecular size. The human placenta is haemomonochorial in structure like the guinea pig, while the rabbit is haemodichorial, the rat haemotrichorial, and the sheep epitheliochorial.

None of the animal placentas studied so far has been shown to have exactly the same permeability characteristics as the human placenta over a wide range of substances. The passive permeabilities of haemochorial placentas as a group are quite similar over the molecular size range of 180 to 5000 and this contrasts quite sharply with the corresponding passive permeability of the epitheliochorial placenta of the

**Placental  
permeability  
(ml sec<sup>-1</sup> g<sup>-1</sup>)**

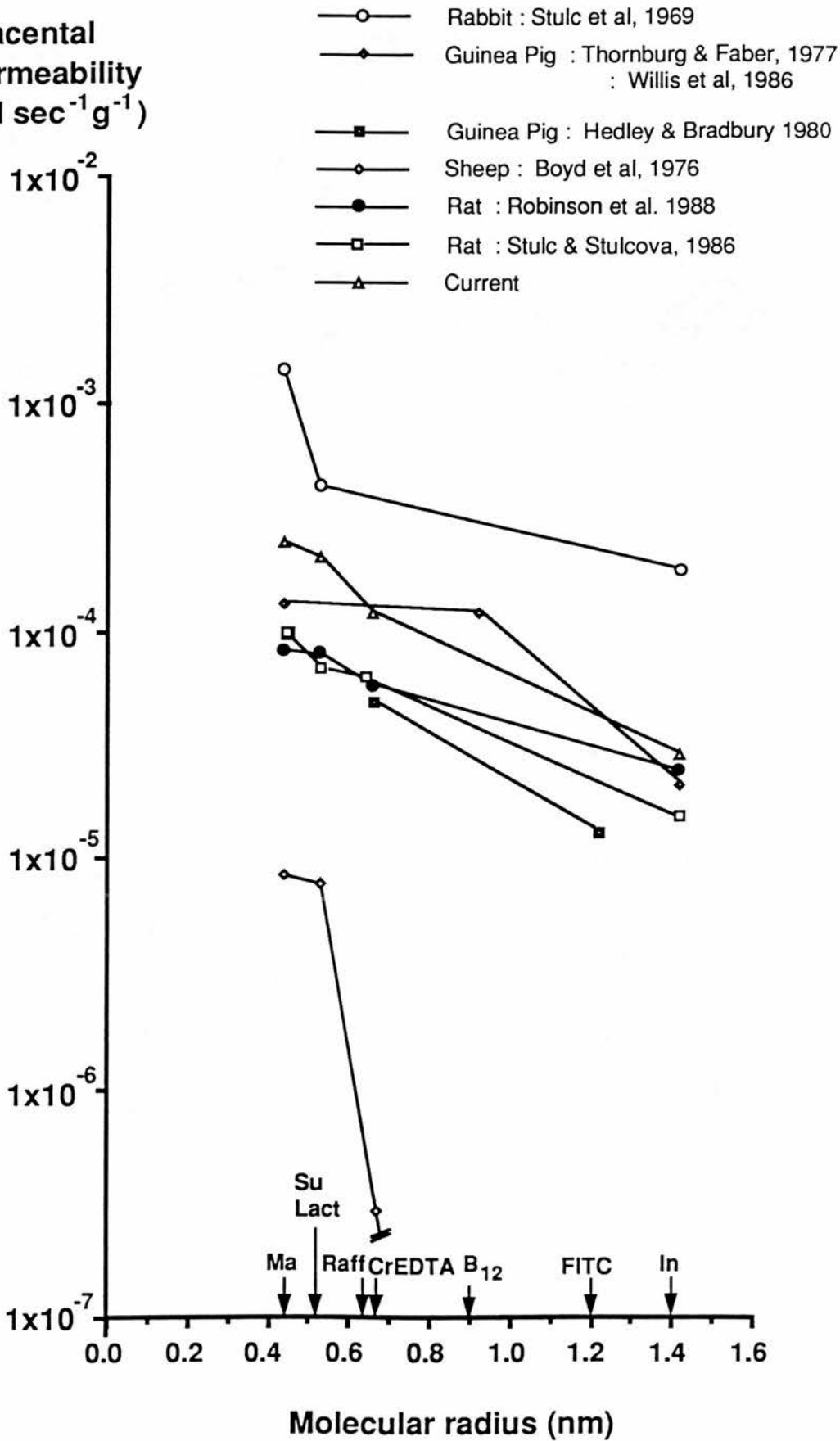


Fig 30. Comparison of the 'in-vivo' human placental permeability data with equivalent data for other animal species. Ma., mannitol; Su., sucrose; Lact., lactulose; Raff., raffinose; B<sub>12</sub>., cobalamin; FITC., fluorescein-labelled dextran, molecular weight 3000; & In., inulin



sheep. A similar variation with placental structure is also found for the placental permeability for sodium (Moll, 1985), but a totally different relationship between placental structure and permeability is revealed by gas transfer across the placenta. Placental diffusing capacity (a measure of permeability to gases) to carbon monoxide is very similar in the rhesus monkey (haemochorial) and sheep (epitheliochorial) but 20 times less than that of the guinea pig (haemochorial) when expressed per unit placental weight (Faber & Thornburg, 1983).

While the differences in passive permeabilities between species are quite obvious, the biological meaning of these differences remains obscure (Moll, 1985). The sheep placenta has a preplacental arterial blood pressure of 80% of central arterial while in the haemochorial placentas examined (Moll, 1985) the preplacental blood pressure varied between 8 to 18%. The tightness of the sheep placenta may reflect this high pressure.

#### Physiological implications of the permeability data.

The 'in-vivo' permeability data for the human placenta derived above allow further understanding of both the nature of the placental barrier and the functional consequences of the barrier as they affect the ability of the placenta to meet the nutritional and excretory requirements of the developing foetus.

#### 1. The nature of the placental barrier.

The placenta is an anatomical barrier that separates the



maternal and foetal vascular compartments, yet has to allow sufficient exchange between these compartments to meet the foetal nutritional and excretory requirements. It is already recognised that this anatomical barrier also provides a barrier to free exchange between mother and foetus (Stacey 1985). Although IgG can cross the placenta, mainly in the third trimester and probably by receptor mediated endocytosis, the much larger immunoglobulin IgM cannot and its presence in the foetus is indicative of foetal production. The early studies by Gitlin and colleagues (Gitlin et al, 1964, and Gitlin & Gitlin, 1975) demonstrated the impermeability of the placenta to quite small proteins by measuring the appearance of radioiodinated proteins in the foetus after injection into the maternal circulation. The transfer rate was found to be very low indeed. Transfer studies with iodinated compounds have had their difficulties, but these results accord with other evidence. Thus neonates born with the rare genetic variant of analbuminaemia would not be born with almost undetectable plasma albumin concentrations were it not for the impermeability of the placenta to albumin. Likewise, congenital nephrotic syndrome would be corrected in utero if the placenta were permeable to albumin.

Johnson et al (1974) examined the discordance between the haplotypes of various plasma proteins in the maternal and foetal circulations. Out of 10 cases examined, there was discordance in four between the maternal and foetal (as determined from cord blood) haplotypes for orosomucoid, a protein with molecular weight 44,000, indicating the

placenta to be impermeable to this protein and by inference, molecules of similar size. In their study with  $^{125}\text{I}$  insulin, molecular weight 5733, Kalhan et al, (1975) found that after 1 hour of a constant rate maternal infusion the umbilical venous count was only 1% of maternal venous blood and concluded that at term there was no significant maternal-foetal transfer in man. Insulin can however cross the placenta to the foetus. A recent study on the placental transfer of insulin in man (Menon, Cohen, Sperling, et al, 1990) for example, demonstrated a relation between the placental transfer of porcine, bovine, or synthetic human insulin and the level of maternal antibody to these insulins, indicative of antibody-mediated transfer. This is analogous to the situation found in the guinea pig placenta where Thorell (Thorell, 1966) demonstrated that insulin crosses the guinea pig placenta bound to maternal antibodies but with no transfer of insulin to the foetus in the absence of maternal antibodies.

The molecular weight of insulin is 5733, only slightly greater than that of the largest permeant, inulin, used in current study and insulin might therefore be expected to cross the placenta if the only criterion were molecular weight. The reason for the measured impermeability of the human placenta to insulin is not yet clear. The plasma half-life of intravenously administered insulin is at most 30 minutes due to rapid clearance by many tissues including the placenta, and a slow transfer, as might be expected from the present data, may remain undetected by the sort of technique used by Kalhan et al (1975). Alternatively, the placental

transfer of insulin may indeed be insignificant and the explanation for its this may lie in the size or shape of the insulin molecule, discrimination by the placenta on the basis of surface charge, the existence of insulin in polymeric forms in solution, or simply very effective placental uptake and metabolism.

The measured clearances for the four permeants indicate that while the human placenta is permeable to molecules up to the size of inulin, somewhere above this molecular size and below that of orosomucoid, this permeability is apparently lost. Further work is obviously required to define the upper limit of placental passive permeability.

A clearance value for inulin of  $0.9 \text{ ml.min}^{-1}$  and  $8 \text{ ml.min}^{-1}$  for mannitol compares with estimates of placental blood flow of  $500 \text{ ml.min}^{-1}$  in the uterine artery and  $300 \text{ ml.min}^{-1}$  in the umbilical artery at term (Faber & Thornburg, 1983). The figures for blood flow correspond to plasma flows of approximately 300 and  $160 \text{ ml.min}^{-1}$  respectively and these are the more relevant flows when considering the passive permeability measured in the current study as the permeants do not enter red cells. The determined clearance values are low when compared to both the maternal and foetal flows, blood or plasma, on either side of the placenta. The clearance values are measures of the permeability of the placenta and cannot be directly compared with the maternal and foetal blood flows; carbon monoxide a small molecule with a high lipid solubility for example, has a clearance in man of  $294.6 \times 10^3 \text{ ml.min}^{-1}$ . The exact relation between the placental clearance and placental blood flows depends on the

precise anatomical relations of the the maternal and foetal blood flows, concurrent, countercurrent, or some other type. Faber and Thornburg (1983) in a mathematical analysis of a variety of placental blood flow models, found that the transfer of substances with placental clearances less than  $66 \text{ ml.min}^{-1}$  was always limited by the permeability of the placenta while for substances with clearances greater than  $4400 \text{ ml.min}^{-1}$  the transfer was not limited by the permeability, that is, was governed by the blood flows on either side of the placenta. Though in the human the relations of the flows is unproven, these calculations by Faber and Thornburg agree with the general belief that the clearances of small hydrophilic molecules will be descriptive of the membrane characteristics compared with the permeability to respiratory gases.

While anatomical shunting (blood not reaching the exchange area) and mismatch (blood flows not matched on either side of the exchange area) of the two circulations occurs in animal placentas it is unlikely to be of the magnitude required to be the sole explanation for the low clearances observed in the human placenta 'in-vivo'. Measurements of shunting have varied from 19% of umbilical flow in the sheep, 23% of umbilical and 36% of uterine flow in the goat, and 2% of umbilical flow in the guinea pig (Faber & Thornburg, 1983). Shunting of these orders of magnitude is insufficient to render the placental transfer of the permeants studied flow limited and the low measured clearances found in the human placenta 'in-vivo' are likely therefore to be a reflection of a significant barrier to

passive diffusion.

Nature of the placental membrane hydrophilic pathways.

The permeability profile of the four permeants allows the placental barrier to hydrophilic substances to be analysed in terms of the pore theory. Boyd et al (1976) performed such an analysis on the 'in-vivo' permeability of the sheep placenta to radiolabelled urea, erythritol, and mannitol, and showed that the data was consistent with diffusion via cylindrical, water-filled pores of a uniform diameter of 0.43 to 0.45 nm. Stulc et al, (1969), had previously analysed similar permeability data (water, urea, erythritol, mannitol, sucrose, inulin and albumin) in the rabbit and found that the data from the rabbit were incompatible with an isoporous model and required at least two distinct populations of different sized pores, a large population of small pores of radius 0.3 nm and a small population of large pores of radius 10 nm. Hedley and Bradbury (1980) reported a similar finding in the guinea pig. Analysing the permeability data for radiolabelled Na, K, erythritol and CrEDTA and native FITC Dextran 3000 and 20000, these authors found the data again required two different populations of pores, one with a radius of 10 nm allowing the passage of CrEDTA and the two Dextran and another with a radius of 0.3 nm allowing the transfer of erythritol, Na, and K. Robinson et al (1988) have recently reported permeability studies in the rat placenta using the same sized permeants (radiolabelled) as the current study, mannitol, sucrose, CrEDTA, and inulin, but with the addition of radiolabelled Na and albumin. These data indicated a single population of

pores allowing the passage of the four intermediate sized permeants mannitol, sucrose, CrEDTA, and inulin, but the transfer of Na required a different route that could represent a second population of smaller sized pores. The similarity between the computed figures for equivalent pore radii in the guinea pig and rabbit is striking.

The current 'in-vivo' permeability data for the human placenta can also be analysed by pore theory to determine whether the 'in-vivo' placental permeability data is compatible with a uniporous model as is the case for the sheep (Boyd et al, 1976) or a non-uniporous or multiporous model as suggested for the rabbit (Stulc et al, 1969), guinea pig (Hedly & Bradbury, 1980), and rat (Stulc, 1989). Back-calculating using the Faxen-Ferry term, the permeability for mannitol is converted into the notional permeability corresponding to unrestricted diffusion through a given pore size. This notional permeability can be used to construct a permeability profile on the basis of unrestricted diffusion, as on a log-log plot of permeability versus molecular radius the slope of this line will be -1. Applying the Faxen-Ferry term to the line so constructed for each pore size, the degree of restricted diffusion to the molecular size of each permeant allows the profile of placental permeability to be determined for different pore radii (Levick, 1991) Fig 31. This analysis was done graphically demonstrating that a single pore radius cannot accomodate all the data; the best fit is with a pore radius of 5 - 6 nm which poorly accomodates the data for CrEDTA. When the data from individual patient-studies are considered

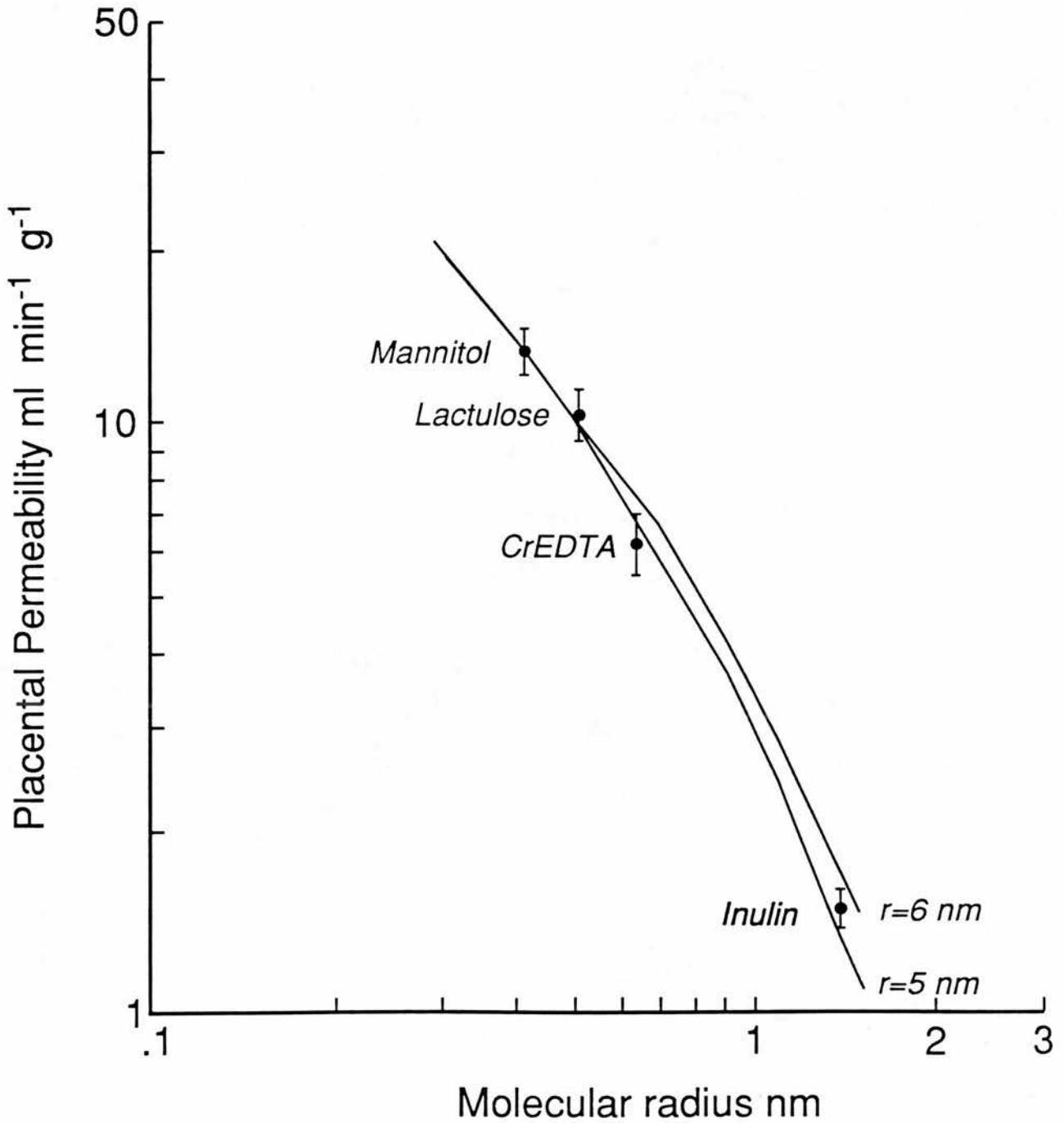


Fig 31. Placental permeability profiles for a single pore radius ( $r$ ) of either 5 or 6 nm calculated with reference to mannitol. The measured 'in-vivo' permeant permeabilities are mean  $\pm$  S.E. of mean



separately they deviate even further from a uniporous model. The permeability data therefore better fit a multiporous model when analyzed terms of pore theory.

Distribution of permeability within the placental barrier.

The site or sites of the barrier to passive diffusion within the placental membrane in the human is as yet unclear. The placental membrane is composed of several different layers that will differ in their permeability properties, and the overall permeability of the membrane will represent the summation of its component layers. It is most unlikely that these characteristics will be vested in one layer alone: classically, epithelia such as the trophoblast are 'tight', restricting transfer by passive diffusion whereas capillary endothelia (with the exception of the brain) are much more permeable allowing the transfer of large molecules such as plasma proteins.

The relative contributions of the individual tissue layers to the overall permeability of the placental membrane to hydrophilic molecules of different size has been studied in the rabbit placenta by Thornburg & Faber (1976). These authors found that more than 90% of the diffusion resistance (diffusion resistance is the inverse of permeability, PS) to small hydrophilic molecules was provided by the inner layer of the trophoblast, while the foetal capillary wall contributed only some 5 - 10 %. The foetal capillary wall did, however, significantly restrict the transplacental diffusion of the large molecule, transferrin (MW  $1 \times 10^6$  daltons), 55% of the resistance residing in the capillary endothelium and 41% in the cytotrophoblast and 4% in the



syncytiotrophoblast. Stulc (1989) has recently analysed the permeability data for the rabbit and rat placenta obtained in his laboratory in terms of two porous membranes in series, a low permeability, large pore membrane representing placental trophoblast, and a high permeability small pore membrane, representing the foetal capillary wall. The fit with this model was very good. In the sheep both the trophoblast and capillary endothelium are tight preventing the transfer of molecules larger than disaccharides (Boyd et al, 1983).

Studies with membrane vesicles demonstrate that the trophoblastic cell membrane of the human placenta is tight and relatively impermeable even to the passive diffusion of small molecules, for example mannitol and sucrose are used as buffers to prevent osmotic changes in placental membrane vesicle preparations (Bissonnette, 1982). The outer layer of the trophoblast is a syncytium, the syncytiotrophoblast, with therefore few if any paracellular channels. All aqueous pores in this layer must therefore be transcellular and large enough to allow the passage of molecules up to the size of inulin. This is in contrast to the absorptive epithelium, the intestinal mucosa (Menzies, 1984), where there is strong evidence of at least 2 populations of different sized pores, the small pores being transcellular and permitting diffusion of molecules up to the size of monosaccharides, and the larger pores paracellular for the transfer of larger molecules.

There are no lymphatics on the foetal side of the placenta and no lymphatic connection between the umbilical

cord and the foetal body (Boyd & Hamilton, 1967). The absence of any lymphatic drainage would imply little lymph production which in turn would require a tight capillary endothelium relatively impermeable to albumin as albumin is the major osmotically active protein in lymph (conc. 15 - 20 g.l<sup>-1</sup> depending on the organ in question, and a slightly higher percentage of the total protein content than in plasma). The lack of a lymphatic drainage system in other animals studied has been associated with a high diffusion resistance to large molecules in the foetal capillary endothelium in the sheep (Boyd et al, 1983) and the guinea pig (Thornburg & Faber, 1976). There is other indirect evidence for a significant contribution to diffusion resistance from the foetal capillary in the human. Proteins produced by the trophoblast itself, such as placental lactogen and placental alkaline phosphatase appear in the maternal circulation in considerable quantities, but do not accumulate in the foetus. On the other hand, proteins produced within the foetus itself such as  $\alpha$ -fetoprotein and growth hormone remain at high concentrations in foetal plasma, but are found at very much lower concentrations in maternal plasma (by four orders of magnitude in the case of  $\alpha$ -fetoprotein) (Stacey, 1990).

## 2. Functional consequences of the placental barrier.

The placental barrier is obviously necessary to maintain the separate integrity of the maternal and foetal circulations and separate the immunologically different mother from her foetus. All exchange between the maternal

and foetal vascular compartments in the human occurs across the placenta. Owens and Robinson (1988) listed the placental characteristics determining foetal-maternal exchange for various substrates, Table 14.

Table 14. Placental characteristics determining foetal-maternal exchange of various substrates.

---

Rates of maternal and foetal placental blood flow
Vascular arrangements of maternal and foetal blood vessels
Permeability of the placental membrane: surface area and physical properties
Mechanisms for carrier-borne transport
Placental utilization

---

The importance of placental permeability to normal foetal growth and development is demonstrated by the way in which all the pertinent properties of the placental membrane that determine its permeability increase with foetal growth. In the sheep, placental clearance of urea increases with gestational age and is proportional to foetal rather than placental weight. A logarithmic increase also occurs in the last third of gestation when placental weight and DNA content are constant but foetal weight is increasing (Moll, 1985). Flexner and Pohl (1941) measured the permeability for sodium in the guinea pig and here too, in spite of a large scatter of the data, there was a proportional relationship between permeability and foetal weight.

There is much experimental evidence, stronger in animals, linking reduced placental transfer with foetal growth, summarized by Owens and Robinson (1988). In the human, chronic maternal undernutrition is associated with reduced placental and foetal weight, and any reduced uterine blood flow is associated with an increased risk of intra-uterine growth retardation. In women living at high altitude there is a decrease in average birth weight that has been interpreted as a response to restricted availability to the foetus of oxygen, a major foetal substrate. The positive association between placental and foetal weight is, however, not strong evidence for substrate limitation of foetal growth as the placenta is a foetal organ and might therefore be expected to parallel any change in the foetus.

In animals, attempts to limit placental function either by reducing placental size or embolization of part of the vascular bed, result in reduced uterine blood flow. In most of these studies, reduced blood flow was correlated with reduced foetal weight and this has been interpreted as the restriction of foetal growth by flow limited transfer rather than membrane limited transfer though other effects were not excluded. Few measurements of passive permeability following manipulation of placental growth have been undertaken. Spontaneously occurring small placentas in sheep have been reported as having normal permeability to urea when expressed as a measure of placental weight, and surgical reduction in placental size in the sheep produced a similar result. Both these observations indicate that placental permeability will decrease with placental size leading to

the possibility that under the right circumstances, placental permeability may become growth limiting.

The lack of correlation in the present study between placental growth and placental clearance indicates that while passive permeability appears to be important for foetal growth (Moll, 1985), at term and in normal pregnancies with birth weights ranging from 2.9 to 4.4 kg, placental permeability does not appear to be limiting foetal growth.

#### Substrate transfer across the placenta.

The determined values for placental clearance are measures of the amount of placental transfer available to the foetus by passive diffusion across the placental barrier. They therefore allow an assessment of whether any special transfer mechanisms are required to meet the foetal requirement or whether passive diffusion alone is sufficient.

Glucose has the same molecular size and lipid solubility as mannitol and will have an identical placental clearance by passive diffusion. The foetal glucose requirements at term have been estimated by several different authors using several different approaches and they all give figures in the range of 4 - 8 mg glucose.kg<sup>-1</sup>.min<sup>-1</sup> (Battaglia & Sparks, 1983). The maternal-foetal concentration gradient has been measured and found to be approximately 0.07 mg.ml<sup>-1</sup> (Oakley, Beard, & Turner, 1972). Using turnover techniques with isotopically labelled 1-<sup>13</sup>[C] glucose, Kalhan and colleagues (1979) were able to demonstrate that, at term but before the onset of labour, the foetus in utero produces no

glucose and that the foetal glucose is entirely maternal in origin. Taking  $0.07 \text{ mg.ml}^{-1}$  as the maternal concentration gradient of glucose and the foetal glucose consumption and applying the equation for the calculation of clearance (p 90), a figure of  $340 \text{ ml.min}^{-1}$  is obtained. This is the notional clearance of glucose that would be required if passive diffusion were the only means of placental transfer and this is 40 times greater than the  $8 \text{ ml.min}^{-1}$  measured for mannitol. Clearly diffusional transfer alone is insufficient to meet the estimated placental transfer rate of glucose and it is necessary to postulate carrier-mediated transfer of glucose. Widdas (1952) first suggested facilitated carrier-mediated transfer for glucose transfer from 'in-vivo' studies in the sheep, but definitive proof was not obtained until 1977, when placental transfer in the chronically catheterised sheep was studied using inert analogues of glucose (Stacey et al, 1977). While L-glucose had a placental clearance approximately similar to that of mannitol, 3-methylglucose had a clearance two orders of magnitude greater and this could only occur if there were carrier-mediated transfer. The rate of transfer provided by the carrier in the sheep is the same order of magnitude as that which would be required in the human placenta.

Carrier-mediated transfer mechanisms have been demonstrated in human 'in-vitro' preparations of the placenta and there is circumstantial evidence for them from 'in-vivo' studies. Thus Cordero et al (1970) studying short glucose infusions in women, found a significant correlation between maternal and foetal levels but with a ceiling or



plateau ( $350 \text{ mg.dl}^{-1}$ ) above which there was no further increase in foetal levels. Oakley (1972) confirmed the above concept of a ceiling for the placental transfer of glucose but found the plateau to be lower at  $200 \text{ mg.dl}^{-1}$ . The existence of a plateau is evidence of saturation and this is one of the hallmarks of carrier-mediated transfer. The current study provides not only further direct evidence for the existence 'in-vivo' of carrier-mediated transfer for glucose, but provides a reason for its necessity.

Data for other important metabolites can be similarly analysed if a measure of foetal flux can be obtained. Urea, molecular weight 60 daltons, has a transplacental fetal-maternal concentration gradient of  $25 \mu\text{g.ml}^{-1}$  (Morriss & Boyd, 1988) and a neonatal production rate estimated at  $200 \text{ mg.kg}^{-1}.\text{day}^{-1}$  for bottle fed infants (Ciba Geigy, 1981). Unfortunately the data available for production rate calculated by Gresham, Simons & Battaglioli (1971) assumed for man the same placental clearance as the Rhesus monkey. Taking the neonatal production rate as an estimate of intra-uterine production, this corresponds to a clearance for urea of  $15 \text{ ml.min}^{-1}$  compared with  $8 \text{ ml.min}^{-1}$  for the measured clearance of mannitol, a molecule twice its size. Assuming a linear extrapolation of the current clearance data, passive diffusion alone could provide for a clearance of  $15 \text{ ml.min}^{-1}$ . Creatinine (MW 116) shows a neonatal production rate of  $1.7 \text{ mg.kg}^{-1}.\text{day}^{-1}$  (breast fed infants) (Ciba Geigy, 1981) and passive diffusion would only require a foetal-maternal concentration gradient of  $1 \mu\text{g.ml}^{-1}$  if placental transfer were by passive diffusion alone. The absence of a

readily identifiable transplacental concentration gradient for creatinine (Morriss & Boyd, 1988) is most likely due to the difficulty of detecting such a small gradient. The ability of the placenta to meet the foetal excretory requirement for creatinine and possibly urea by passive diffusion alone would explain the absence to date of any identifiable carrier-mediated transport mechanism for the transfer of these substrates across the placenta.

In congenital hypothyroidism, the opposite situation appears to pertain as passive diffusion cannot compensate for foetal under-production of thyroid hormones. Infants born at birth with this condition show evidence of intra-uterine thyroxine deficiency at birth, with abnormal epiphyses and delayed ossification so that clearly the placental clearance of thyroid hormones cannot compensate for reduced foetal production. The neonatal replacement dose of thyroxine of  $25 \mu\text{g} \cdot \text{day}^{-1}$  can be taken as an approximation of foetal requirement. In the case of complete absence of the thyroid where endogenous foetal production and levels will be zero, the maternal levels of free plasma thyroxine of  $13 \text{ pg} \cdot \text{ml}^{-1}$  and free tri-iodothyronine of  $5 \text{ pg} \cdot \text{ml}^{-1}$  (Kendall-Taylor, 1978) would require a passive clearance of approximately  $1000 \text{ ml} \cdot \text{min}^{-1}$ . This is three orders of magnitude greater than the combined clearance by passive diffusion of both forms of the thyroxine based on molecular size, which is intermediate between that of CrEDTA and inulin, that is  $1 - 4 \text{ ml} \cdot \text{min}^{-1}$ . Without carrier-mediated transport for thyroxine, for which there is no evidence, placental transfer cannot ameliorate the consequences of



congenital hypothyroidism by means of maternally derived thyroid hormone.

#### Foetal Homeostasis.

The placenta does act as a significant barrier to the free diffusion of substrates between mother and foetus. This would have the advantage of allowing foetal homeostasis to a greater or lesser extent to be independent of that of the mother allowing the foetus for example, to accumulate and retain iron even in the face of maternal deficiency with the result that iron deficiency is virtually unknown in the newborn. This would be of evolutionary advantage as although maternal iron deficiency is rare in the developed world with a good diet and small families, it is a common finding in the developing world and is likely to have been a common feature of evolutionary life. Moll (1985), also stresses the evolutionary advantage of independent foetal homeostasis protecting the foetus from transient harmful concentrations of maternal metabolites that accumulate under stress, such as during fight, flight, and fright. Thus transient maternal lactic acidosis occurring in a cow has little impact on its foetus because of the low placental permeability.

The degree of homeostatic independence of the mother thus afforded by the placental barrier is however incomplete, and not always beneficial. The placenta is unable to protect the foetus from osmotic fluid shifts originating in the mother. It is now well recognised that foetal hyponatraemia can easily develop when the mother is given, not always large, amounts of intravenous fluids of effectively low osmolality (dextrose solutions) during the management of labour. The

hyponatraemia can be quite severe indeed (Stacey, 1990).

Foetal independence due to low placental permeability may thus be achieved at the cost of some restriction in the free transfer of nutrients and waste products between mother and foetus.

## Chapter 7.

## Conclusions.

The technique of measuring the 'in-vivo' permeability of the human placenta described in this study provides direct measurements of one of the most important functions of the placenta, namely transfer between the maternal and foetal compartments. Free diffusion is the simplest form of transfer across biological membranes and is common to all hydrophilic placental permeants. The only other direct measure of placental transfer currently available is foetal growth itself. All the other currently available measures of so-called placental function concern other functions of the placenta which are not directly related to nutrition of the foetus, for example urinary oestriol excretion.

The measurements made in this study demonstrate that the passive permeability of the human placental membrane is low and that it presents a significant barrier to free diffusion between maternal and foetal vascular compartments. This means that for hydrophilic metabolites such as glucose and amino acids, special transfer mechanisms are required to meet foetal requirements as these latter exceed the rather low limits of passive transfer imposed by the permeability characteristics of the placenta. It would thus appear that for glucose and probably amino acids, most of the placental transfer occurs via carrier-mediated mechanisms and free

diffusion makes little contribution to the net transfer; for glucose some 3% of net foetal requirement appears to be met by passive diffusion. Although not quantitatively important for glucose and amino acid transfer, other experimental evidence indicates that passive permeability is closely correlated with foetal weight, and that this correlation is not solely due to an increase in placental surface area as it occurs despite a lag of chorionic surface area behind foetal weight (Moll, 1985), implying a simultaneous increase in membrane permeability. This precise adjustment of placental permeability to foetal growth implies a functional significance which may lie in foetal excretion rather than foetal nutrition as free diffusion appears to be the only mechanism for urea and creatinine excretion by the foetus.

Appreciation of placental permeability is important with the increasing clinical intervention and use of pharmaceuticals during pregnancy. A knowledge of the likely transfer across the placenta of any agents so used will allow prediction of their effect on the foetus. Thus reference has already been made to the rapid fluid shifts which can occur across the placenta and the caution which is now used when fluids are administered during pregnancy. The permeability of the placenta to molecules as large as inulin indicates how easily hydrophilic agents can enter the foetal compartment. Digoxin (MW 800) can cross the placenta and must therefore be used with caution in pregnancy, but this same transfer can be used therapeutically to control intra-uterine arrhythmias (Wladimiroff JW & Stewart PA, 1985).

The current research has highlighted the difficulties in

choosing a suitable animal model for human placental function as a similarity between the model and the human placenta for one set of physiological characteristics does not imply a corresponding similarity between any other sets. 'In-vitro' perfusion of the human placenta is also shown to reproduce poorly, if at all, the passive permeability characteristics found 'in-vivo'. Attention had focused in the past on these animal models and 'in-vitro' studies as a source of data relevant to the human because of the difficulties in undertaking 'in-vivo' measurements in the placenta of physiological and functional characteristics. There is now developing the methodology for making other measurements of physiological parameters of the placenta 'in-vivo' (Chien, Taylor, Scrimgeour et al, 1991). The current study is in the vanguard of this developing field which is all the more important given the limitations of animal models and 'in-vitro' perfusion.

The placental permeability data obtained in the current study form the basis from which to proceed to study placental transfer in pregnancies complicated by disordered foetal growth, for example hypertensive disease of pregnancy and idiopathic growth retardation, which is suspected in part or in whole, to be due to reduced availability of placental exchange to the foetus.

# References.

- Aherne W., and Dunnill MS., (1966). Quantitative aspects of placental structure. *J Path Bact*: 91; 123 - 139.
- Battaglia FC., and Sparks JW., (1983). Perinatal nutrition and metabolism. In *Perinatal Medicine, Butterworths International Medical Reviews, Pediatrics-2*, eds. Boyd RDH., and Battaglia FC p 153. London: Butterworth and Co Ltd.
- Basso A., Fernandez A., Althabe O., Sabini G., Piriz H., and Belitzky R., (1977). Passage of mannitol from mother to amniotic fluid and fetus. *Obstet Gynecol*: 49; 628-631.
- Beach RC., and Menzies., (1983). Lactulose and other non-absorbable sugars in infant milk feeds. *Lancet*: i; 425 - 426.
- Bickel H., (1961). Mellituria, a paper chromatographic study. *J Pediatr*: 59; 641 - 656.
- Bissonnette JM, (1981). Placental transport of carbohydrates. *Mead Johnson Symp. Perinat. Dev. Med*: 18; 21 - 23.
- Bissonnette JM, (1982). Membrane vesicles from trophoblast cells as models for placental exchange studies. *Placenta*: 3; 99 - 106.
- Bittman R, and Blau L, (1972). The phospholipid-cholesterol interaction kinetics of water permeability in liposomes. *J Membr Biol*: 58; 75 - 80.
- Boyd CAR., (1981) Experimental design in transfer experiments. In *Placental Transfer: methods and Interpretations*, Ed. Young M., Boyd RDH., Longo LD &

Telegdy G., pp 69 - 78. London: WB Saunders Co Ltd.

Boyd JD., and Hamilton WH., (1967). Development and structure of the human placenta from the end of the 3rd month of gestation. J Obstet Gynaecol Br Commonw: 74; 161 - 226.

Boyd JD., and Hamilton WJ., (1970). The Human placenta. Cambridge: Heffer and Sons Ltd.

Boyd RDH., Canning JF., Stacey TE., Ward RHT., and Weedon AP., (1983). Volumes of distribution of sodium and albumin in the sheep placenta. J Physiol (Lond): 336; 13 - 26.

Boyd RDH., Haworth C., Stacey TE., and Ward RHT., (1976). Permeability of the sheep placenta to unmetabolized polar non-electrolytes. J Physiol (Lond): 256; 617 -634.

Brandes JM., Abramovici H., and Reich M., (1970). Passage of inulin from mother to fetus as an unchanged polymer. Isr J Med Sci: 6; 734 - 736.

Brandes JM., Abramovici H., and Peretz A., (1971). Passage of inulin from Gravida to Fetus in Normal Pregnancy and During Cesarean Section. Obstet Gynecol: 37; 877 -880.

Campbell S., Wladimifoff JW., and Dewhurst CJ., (1973). The antenatal measurement of fetal urine production. J Obstet Gynecol Br Commonw: 80; 680 - 686.

Cassady G, (1970). Bromide space studies in infants of low birth weight. Pediatr Res: 4; 14 - 24.

Chalmers RA., and Lawson Am., (1982). Organic Acids in Man. London: Chapman and Hall.

Chien PWF., Taylor DJ., Scrimgeour CM., Watt PW., and Rennie MJ., (1991). Whole body protein turnover in the human



- term foetus measured measured at elective caesarean delivery. Abstract presented to the Physiology Society Meeting at University College London, 25 - 26 March 1991.
- Ciba Geigy., (1981). In Geigy Scientific Tables, 8th edn., vol. 1. ed. Lentner C. Basle, Switzerland: Ciba Geigy Ltd.
- Collander R., (1954). The permeability of Nitella cells to non-electrolytes. *Physiol Plant*: 7; 420 - 445.
- Coulthard MG., and Ruddock V., (1983). Validation of inulin as a marker for glomerular filtration in preterm babies. *Kidney Int*: 23; 407 - 409.
- Davison JM., and Hytten FE., (1974). Glomerular filtration during and after pregnancy. *J Obstet Gynaecol Br Commonw*: 81; 588 - 595.
- Davson H., and Danelli JF., (1952). The permeability of natural membranes, 2nd edn. Cambridge: Cambridge University Press.
- Dean RB., (1941). Theories of electrolyte equilibrium in muscle. *Biol Symposia*: 3; 331 - 348.
- Evans WH., and Graham JM., (1989). Membrane structure and function. Oxford: IRL Press.
- Faber JJ., and Thornburg KL., (1983). In *Placental Physiology*. New York: Raven Press.
- Firth JA., Bauman KF., and Sibley CP., (1983). The Intercellular Junctions of Guinea-pig Placental Capillaries: A Possible Structural Basis for Endothelial Solute Permeability. *J Ultrastr Res*: 85; 45 - 57.
- Flexner LB., Cowie DB., Hellman LM., Wilde WS., and Vosburgh GJ., (1948). The permeability of the human placenta to



- sodium in normal and abnormal pregnancies and the supply of sodium to the human fetus as determined with radioactive sodium. *Am J Obstet & Gynecol*: 55; 469 - 480.
- Flexner LB., and Pohl HA., (1941). Transfer of radioactive sodium across the placenta of the guinea pig. *Am J. Physiol*: 132; 594 - 606.
- Fox H., (1979). The correlation between placental structure and transfer function. In *Placental Transfer*, ed. Chamberlain GVP., & Wilkinson AW. Tunbridge Wells: Pitman Medical Publishing.
- Gitlin D., and Gitlin J., (1975). Fetal and neonatal development of human plasma proteins. In *The Plasma Proteins*, Vol. 2, 2nd edn. ed Putnam FW. New York: Academic Press.
- Gitlin D., Kumate J., Urrusti J., and Morales C., (1964). The selectivity of the human placenta and the transfer of proteins from mother to fetus. *J. Clin. Invest*: 43; 1938 - 1951.
- Gresham EL., Simons PS., and Battaglia FC., (1971). Maternal-fetal urea concentration difference in man: metabolic significance. *J Pediatr*: 79; 809 - 811.
- Grosser O., (1927). *Fruhentwicklung, Eihautbildung und Placentation des Menschen und der Saugetiere*. Deutsche Frauenheilkunde, Bd 5. Munich: JF Bergmann.
- Hedley R., and Bradbury MWB., (1980). Transport of Polar Non-electrolytes Across the Intact and Perfused Guinea-pig Placenta. *Placenta*: 1; 277 - 285.
- Heyrovsky A., (1956). A new method for the determination of inulin in plasma and urine. *Clin Chim Acta*: 1; 470 - 474.

- Holmberg NG., Kaplan B., Karvonen MJ., Lind J., and Malm M., (1956). Permeability of placenta to glucose, fructose, and xylose. *Acta Physiol Scand*: 36; 291 - 299
- Hytten FE., (1979). Water transfer. In *Placental Transfer*, ed. Chamberlain GVP., & Wilkinson AW. Tunbridge Wells: Pitman Medical Publishing.
- Illsley NP., Aarnoudse JG., Penfold P., Bardsley SE., Coade SB., Stacey TE., and Hytten FE., (1984). Mechanical and metabolic viability of a placental perfusion system in vitro under oxygenated and anoxic conditions. *Placenta*: 5; 213 - 225.
- Johnson AM., Umansky I., Alper CA., Everett C., and Greenspan G., (1974). Amniotic fluid proteins: Maternal and fetal contributions. *J Pediatr*: 84; 588 - 593.
- Johnson LW., and Smith CH., (1980). Monosaccharide transport across microvillous membrane of human placenta. *Am J Physiol*: 238; C160 - 168.
- Johnson PM., and Brown PJ., (1981). Fc Receptors in the human placenta. *Placenta*: 2; 355 - 370.
- Kalhan SC., D'Angelo LJ., Savin SM., and Adam PAJ., (1979). Glucose production in pregnant women at term gestation. *J Clin Invest*: 63; 388 - 294.
- Kalhan SC., Schwartz R., and Adam PAJ., (1975). Placental barrier to human insulin-I<sup>125</sup> in insulin-dependent diabetic mothers. *J Clin Endocrinol Metab*: 40; 139 - 142.
- Kendall-Taylor P., (1978). Thyroid function and disease. In *Recent advances in endocrinology and metabolism*, Number 1, ed. O'Riorden JLH p 46. London: Churchill Livingstone.
- Labarre CA., (1988). Acute atherosclerosis. A histopathological

hallmark of immune aggression. Placenta: 9; 95 - 108.

Laker MF., (1979). Estimation of disaccharides in plasma and urine by gas-liquid chromatography. J Chromatogr: 163; 9 - 18.

Laker MF., and Mount JN., (1980). Mannitol Estimation in Biological Fluids by Gas-Liquid Chromatography of Trimethylsilyl Derivatives. Clin Chem; 26; 441 - 443.

Landon MJ., Bain MD., Copas DK., and Stacey TE., (1991). Passive permeability of the human placenta: a comparison of 'in-vitro' and 'in-vivo' data. Abstract presented to the Physiology Society Meeting at University College London, 25 - 26 March 1991: manuscript in preparation

Laurent TC., and Killander J., (1964). A theory of gel filtration and its experimental verification. J Chromatogr: 14; 317 - 330.

Lawn AM., Chiquoine AD., and Amoroso EC., (1969). The development of the placenta in the sheep and goat: an electron microscope study. J Anat; 105: 557- 578.

Levick JR., (1991). An Introduction to Cardiovascular Physiology. Butterworth & Co Ltd: London.

Lind T., (1981) Amniotic fluid. In Geigy Scientific Tables, 8th edn, vol. 1., ed. Lentner C. Basle, Switzerland: Ciba Geigy Ltd.

Ludwig H., (1974). Surface structure of the human placenta. In The Placenta: Biological and Clinical Aspects, Ed. Moghissi KS., & Hafez ESE. Illinois: C.C. Thomas.

Matre R., and Johnson PM., (1977). Multiple Fc receptors in the human placenta. Acta Pathol Microbiol Scand: 85; 314 - 316.

- Maxton DG., Bjarnason I., Reynolds AP., Catt SD., Peters TJ., and Menzies IS., (1986). Lactulose  $^{51}\text{Cr}$ -labelled ethylenediaminetetra-acetate, L-rhamnose and polyethyleneglycol 500 as probe markers for assessment 'in-vivo' of human intestinal permeability. Clin Sci: 71; 71 - 80.
- Menzies IS (1984). Transmucosal passage of inert molecules in health and disease. In Intestinal absorption and Secretion, Ed. Skadhauge E., and Heintze K. Boston: MTP Press.
- Menon RK., Cohen RM., Sperling MA., Cutfield WS., Mimouni F., and Khoury JC., (1990). Transplacental passage of insulin in pregnant women with insulin - dependent diabetes mellitus. N. Engl. J. Med.: 323; 309 - 315.
- Michel CC, (1980). Filtration coefficients and osmotic reflexion coefficients of the walls of the single frog mesenteric capillaries. J Physiol (Lond): 309; 341-355.
- Moll W., (1981). Diffusional placental transfer. In transfer across the primate and non-primate placenta, Ed Wallenburg HCS., van Kreel BK., & van Dijk JP. Placenta: Supplement 1. London; W.B. Saunders Co Ltd.
- Moll W., (1985). Physiological aspects of placental ontogeny and phylogeny. Placenta: 6; 141- 154.
- Morriss FH., and Boyd RDH., (1988). Placental transfer. In The Physiology of Reproduction, Ed Knobil E., & Neill JD. New York: Raven Press.
- Normand ICS., Olver RE., Reynolds EOR., and Strang LB., (1971). Permeability of lung capillaries and alveoli to

non-electrolytes in the fetal lamb. *J Physiol (Lond)*: 219; 303 - 330.

Oakley NW., Beard RW., and Turner RC., (1972). Effect of sustained maternal hyperglycaemia on the fetus in normal and diabetic pregnancies. *Br Med J*: 1; 466 - 469.

Owens JA., and Robinson JS., (1988). The Effect of Experimental Manipulation of Placental Growth and Development. In *Perinatal Practice, Volume 5; Fetal and Neonatal growth*, Ed Cockburn F. Chichester: Wiley Medical Publications.

Pearse JM., and Steel SA., (1988). Use of Doppler ultrasound to investigate the uteroplacental and fetal circulations. *Hospital Update*: 14; 1094 - 1106.

Pharmacia., (1985). Gel filtration: theory and practice. Lund, Sweden: Rahms.

Pitkanen E., (1972). The serum polyol pattern and the urinary polyol excretion in diabetic and in uraemic patients. *Clin Chim Acta*: 38; 2221 - 230.

Robinson NR., Atkinson DE., Jones CJP., and Sibley CP., (1988). Permeability of the Near-term Rat Placenta to Hydrophilic Solutes. *Placenta*: 9; 361 - 372.

Rodbard D., (1976). Estimation of molecular weight by gel filtration and gel electrophoresis. In *Methods of protein separation, Vol. 2*, ed. Catsimpoolas N. New York: Plenum Press.

Schneider H., Sodha RJ., Proegler M., and Young MP., (1985). Permeability of the human placenta for hydrophilic substances studied in the isolated dually perfused lobe. *Contrib Gynecol Obstet*: 13; 98 - 103.

Seller M., & Cole KJ., (1980). Polyacrylamide gel electrophoresis of amniotic fluid cholinesterases: a good prenatal test for neural tube defects. *Br J Obstet Gynaecol*: 87; 1103 - 1108.

Smith CH., (1981) Incubation techniques and investigation of placental transport mechanisms 'in vitro'. In *Placental Transfer: Methods and Interpretations*, Ed. Young M., Boyd RDH., Longo LD & Telegdy G., pp 69 - 78. London: WB Saunders Co Ltd.

Smith CH., and Depper R., (1974). Placental amino acid uptake II. Tissue preincubation, fluid distribution, and mechanisms of regulation. *Pediatr Res*: 8; 697 - 703.

Snedecor GW., and Cochran WG., (1980). *Statistical Methods*, 7th edn. Iowa: Iowa State University Press.

Solomon AK., (1968). Characterization of biological membranes by equivalent pores. *J Physiol (Lond)*: 51; 335 - 364s.

Stacey TE., (1985). Effect of mutation on maternal-fetal metabolic homeostasis: general concepts. In *Genetic and Metabolic Disease in Paediatrics*, ed. Lloyd JK & Scriver CR. London: Butterworths International Medical Reviews.

Stacey TE., (1990). Placental transfer. In *Clinical Physiology in Obstetrics*, 2nd edn, Ed Chamberlain GVP & Hytten FE. Oxford: Blackwell Scientific Publications Ltd.

Stacey TE., Boyd RDH., Ward RHT., & Weedon AP., (1977). placental permeability in the sheep. *Ann Rech Vet*: 8; 345 - 352.

Stacey TE., Weedon AP., Haworth C., Ward RHT., & Boyd RDH., (1978). Fetomaternal transfer of glucose analogues by

- sheep placenta. *Am J Physiol*: 234(1); E32 - E37.
- Stulc J., (1989). Extracellular transport pathways in the haemochorial placenta. *Placenta*: 10; 113 - 119.
- Stulc J., Friedrich R., and Jiricka Z., (1969). Estimation of the equivalent pore dimensions in the rabbit placenta. *Life Sci*: 8; 167 - 180.
- Stulc J., and Stulcova B., (1986). Transport of calcium by the placenta of the rat. *J Physiol (Lond)*: 371; 1 - 16.
- Super M., Fennell SJ., Schwartz M., and Boyd RDH., (1986). Investigation of cholinesterase in amniotic fluid. *Br Med J*: 293; 17 - 18.
- Teasdale F., and Jean-Jacques G., (1988). Intrauterine growth retardation: morphometry of the microvillous membrane of the human placenta. *Placenta*: 9; 47 - 55.
- Taylor A., and Green P., (1988). Determination of chromium in serum by electrothermal atomisationatomic absorption spectrometry using totally pyrolytic graphite cuvettes. *J Anal At Spectrom*: 3; 115 - 118.
- Thorell JI., (1966). Placental transfer of insulin-131-I in guinea pigs immunized against insulin. *Acta Endocrinol*: 52; 276 - 291.
- Thornburg KL., Burry KJ., Adams AK., Kirk EP., and Faber JJ., (1988). Permeability of placenta to inulin. *Am J Obstet Gynecol*: 158; 1165 - 69.
- Thornburg KL., and Faber JJ., (1977). Transfer of hydrophilic molecules by placenta and yolk sac of the guinea pig. *Am J Physiol*: 233; C111 - C124.
- Venugopal B., and Luckey TD., (1978). Metal toxicity in mammals, Volume 2. toxicity of metals and metalloids. New



York: Plenum Press.

Weaver LT., Laker MF., and Nelson R., (1984). Intestinal permeability in the newborn. Arch Dis Child: 59; 236-241.

Weedon AP., Stacey TE., Ward RHT., and Boyd RDH., (1978). Bidirectional sodium fluxes across the placenta of conscious sheep. Am J Physiol: 235; 536 - 541.

Widdas WF., (1952). Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. J Physiol (Lond): 118; 23 - 39.

Willis DM., O'Grady JP., Faber JJ., and Thornburg KL., (1986). Diffusion permeability of cyanocobalamin in the human placenta. Am J Physiol: 250; R459 - R464.

Wladimiroff JW., and Stewart PA., (1985). Treatment of fetal cardiac arrhythmias. Br J Hosp Med: 34; 134 - 140.

#### Bibliography for Chapter 1.

Faber JJ., and Thornburg KL., (1983). Placental Physiology. New York: Raven Press.

Morriss FH., and Boyd RDH., (1988). Placental transfer. In The Physiology of Reproduction, Ed Knobil E., & Neill JD. New York: Raven Press.

Patten BM., (1953). Human embryology, 2nd Edn. Tokyo: McGraw-Hill Book Co. Ltd, Inc.

Poynter FNL., Ed., (1963). Chemistry in the Science of Medicine. Pitman Medical Publishing Co. Ltd: London.

Steven DH., Ed., (1975). Comparative Placentation: Essays in Structure and Function. London: Academic Press.